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COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

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Vol. 21 No. 3

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A C T A B I O C H I M I C A P O L O N I C A

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*The 30th anniversary of Polish People Republic is a mile-stone in one's life, challenging for reflections on the past and the future.*

*After the World War there were only few biochemists left in the country, but Polish Biochemistry survived thanks to its solid roots, which are linked with such names as Marcel Nencki, Leon Marchlewski, Jacob K. Parnas, Kazimierz Białaszewicz and others.*

*The postwar development of biochemistry is due largely to the favourable atmoshpere created, and the attention devoted to the development of science in general, particularly by the Polish Academy of Sciences. Plans for development of new laboratories and adequate research programs, and plans for education and training of a new generation of biochemists were developed in a spirit of mutual understanding and cooperation.*

*There were periods when conditions were unfavourable for the development of sciences in several fields, but happily enough biochemistry was not affected. So it developed rapidly in a friendly contact and collaboration with world biochemistry in general.*

*To-day — under conditions more favourable for scientific research than ever before — the veterans of Polish biochemistry can look to the future with assurance. Retiring from active life, they will have left behind 104 professors and 110 docents of biochemistry, and a Polish Biochemical Society with 1025 members — engaged in research in the interest of our country and Mankind in general.*

*Józef Heller*



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ACTA BIOCHIMICA POLONICA
Vol. 21 1974 No. 3

A. DŻUGAJ, W. WNUK and T. BARANOWSKI

# PURIFICATION AND PROPERTIES OF HUMAN MUSCLE ALDOLASE

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1. Fructose 1,6-diphosphate (FDP) aldolase (EC 4.1.2.13) has been isolated from human muscle and crystallized. The enzyme was homogeneous on ultracentrifugation, polyacrylamide-gel electrophoresis and cellulose-acetate electrophoresis. 2. The specific activity of the enzyme with FDP was about 16 i.u./mg protein and the FDP: F-1-P activity ratio close to 48. 3. The molecular weight of the enzyme was determined by the methods of: sedimentation and diffusion, sedimentation and viscosity, and gel filtration, to be 160 000. 4. By means of carboxypeptidase A digestion and hydrazinolysis, tyrosine has been found to be the COOH-terminal amino acid. The enzyme contains 28 sulphydryl groups and is composed of two types of subunits differing in electrophoretic mobility. The peptide map of human muscle aldolase obtained after tryptic digestion is similar to that of aldolase from rabbit muscle.

Two major classes of FDP-aldolases can be distinguished in biological systems. Aldolases of class I have been found in animals, plants, protozoa and algae; aldolases of class II in bacteria, fungi and blue-green algae (Rutter, 1964). Aldolases of both classes occur in Euglena and Chlamydomonas (Russel & Gibbs, 1967). In vertebrate tissues, three aldolases of class I differing in antigenic, catalytic and electrophoretic properties have been detected (Penhoet *et al.*, 1969b; Lebherz & Rutter, 1969) and termed aldolases A, B and C. Aldolase A is the enzyme characteristic for muscle (Taylor *et al.*, 1948), aldolase B predominates in liver (Rutter *et al.*, 1968), while aldolase C has been isolated from brain tissues (Penhoet *et al.*, 1969b). Five-membered systems A-B, A-C and B-C have been detected in some mammalian tissues (Lebherz, 1972).

Human muscle aldolase was first isolated by Ikehara *et al.* (1969) who used the modified method of Taylor *et al.* (1948). The enzyme showed low specific activity (1.3 i.u./mg protein). From the paper of Penhoet *et al.* (1969a) it can be concluded that preparations obtained by the method of Taylor *et al.* (1948) were inhomogeneous.


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The present paper describes isolation and purification of FDP-aldolase A from human muscle by the method of Penhoet *et al.* (1969a). The enzyme was obtained in crystalline form and found to be homogeneous on ultracentrifugation, polyacrylamide-gel electrophoresis and cellulose-acetate electrophoresis. Specific activity, the pH activity profiles, electrophoretic and hydrodynamic properties, as well as the peptide map of the human enzyme are similar to those of FDP-aldolase from rabbit muscle. The preliminary report of this work has been presented (Dżugaj *et al.*, 1971).

## MATERIALS

Fructose-1-phosphate, sodium salt; fructose-1,6-diphosphate, sodium salt; DFP[1]-treated carboxypeptidase A; NAD, and NADH were obtained from Sigma Chemicals Co. (St. Louis, Mo., U.S.A.). Glycerol phosphate dehydrogenase - triosephosphate isomerase mixture was from Boehringer-Mannheim Corp. (Mannheim, West Germany). Cellulose acetate electrophoresis strips were purchased from Gelman Instrument Comp. (Ann Arbor, Mich., U.S.A.). Iodoacetic acid was obtained from British Drug Houses (Poole, Dorset, U.K.) and purified by recrystallization from light petroleum (b.p. 80 - 100°C). Urea was purified by recrystallization from ethanol. Phosphocellulose was obtained from Whatman Biochemicals Ltd (Maidstone, U.K.). Sephadex G-25 and G-200 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Dowex 50 WX8 ($H^+$) was obtained from Serva (Heidelberg, West Germany). All other compounds were of reagent grade and used without further purification.

Human muscle was obtained by autopsy.

## METHODS

Aldolase activity was assayed in the reaction mixture (Warburg & Christian, 1943) containing: 0.1 mM-Tris-HCl, pH 7.5; 0.21 μM-NAD; 2.5 μM-FDP; 0.03 μM-$Na_2HAsO_4$ and 100 μg of glyceraldehyde-3-phosphate dehydrogenase. To the mixture, an appropriate amount of the aldolase was added.

For determining the FDP:F-1-P activity ratio, the procedure recommended by Blostein & Rutter (1963) was used. The reaction mixture contained 0.1 M-Tris-HCl, pH 7.5, instead of glycylglycine buffer, and the following components: 2 mM-FDP or 10 mM-F-1-P, 30 mM-NADH, 15 μg of α-glycerolphosphate dehydrogenase - triose phosphate isomerase, and 0.08 to 0.16 unit of aldolase activity. All assays were performed with Unicam SP 800 recording spectrophotometer at 25°C.

Protein concentration was determined by the spectrophotometric method of Warburg & Christian (1942). Concentrations of purified aldolase were determined spectrophotometrically using the extinction coefficient of 0.91 according to Baranowski & Niederland (1949).

[1] Abbreviations: DFP, diisopropylfluorophosphate; TPCK, (tosylamido-2-phenyl)ethyl chloromethyl ketone; PCMB, *p*-chloromercuribenzoate.

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*Enzyme purification.* Aldolase from human muscle was isolated by the procedure developed by Penhoet *et al.* (1969a) for the enzyme from rabbit muscle.

*Cellulose-acetate electrophoresis.* Zone electrophoresis on cellulose polyacetate strips was performed in 0.05 M-barbital buffer, pH 8.6, containing 0.01 M-2-mercaptoethanol, at 250 V for 90 min at 6°C. The point of application of the enzyme was equidistant from both electrodes. Aldolase activity was located by the staining technique of Penhoet *et al.* (1966), and protein with Amido Black.

*Polyacrylamide-gel electrophoresis.* Disc-gel electrophoresis was carried out according to Davis (1964). Gels were prepared at 7.5% monomer concentration in Tris-glycine buffer, pH 8.6, and stained with 1% Amido Black in 7% acetic acid. Disc-gel electrophoresis was performed also in the polyacrylamide gel containing 8 M-urea at pH 9 in Tris-glycine buffer.

*Peptide maps.* Chromatography and electrophoresis of aldolases from human and rabbit muscle, digested with TPCK-trypsin were run as described by Kochman & Rutter (1968).

*Carboxypeptidase digestion* — for determining the loss of initial activity with FDP. Aldolase was dissolved in 0.1 M-Tris-HCl - 1% LiCl (pH 7.5) and was incubated at 37°C with a 1:300 molar ratio of carboxypeptidase A to aldolase. Aliquots were removed from the reaction mixture and assayed spectrophotometrically.

*Carboxypeptidase digestion* — for determining the carboxyterminal amino acid. The aldolase was digested with carboxypeptidase A in 0.2 M-$NH_4HCO_3$, pH 8.2, at 37°C using a 500:1 aldolase to carboxypeptidase molar ratio. After a 10 min digestion the released amino acids were absorbed on Dowex WX8 ($H^+$), then eluted with 5 M-$NH_4OH$, freeze-dried and identified by means of the paper chromatography technique in butanol - acetic acid - water (4:1:5, by vol.).

*Hydrazinolysis.* The carboxy-terminal amino acid was determined by the method of Akabori *et al.* as described by Fraenkel-Conrat & Chun Ming Tsung (1967). After exposure to anhydrous hydrazine at 80°C for 24 h, hydrazine was removed, the residue was dissolved in water and hydrazides were separated on Amberlite IRC-50 ($H^+$). The aqueous solution was freeze-dried, dissolved in 0.1 M-HCl and identified by paper chromatography.

*Determination of sulphydryl groups.* The number of sulphydryl groups was determined according to Boyer (1954). Titration with PCMB was performed in 4 M-urea - 0.1 M-Na-phosphate buffer, pH 8.

*Molecular weight determination.* Thin-layer chromatography was used (Morris, 1964) with Sephadex G-200 equilibrated with 0.1 M-Tris-HCl - 0.2 M-NaCl, pH 8.0. The following proteins were used as standards: rabbit muscle aldolase prepared according to Penhoet *et al.* (1969a), mol. wt. 160 000; bovine serum albumin, mol. wt. 69 000; glyceraldehyde-3-phosphate dehydrogenase from human muscle prepared according to Baranowski & Wolny (1963), mol. wt. 144 000; enolase from human muscle obtained after Baranowski *et al.* (1968), mol. wt. 90 000.

*Hydrodynamic properties.* Sedimentation-velocity experiments were performed in the MOM analytical ultracentrifuge equipped with phase-plate schlieren optics and a rotor temperature control unit. All runs were carried out at 50 000 rev./min

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(142 000 *g*) at 20°C. Diffusion was studied in a free electrophoresis apparatus (type LKB 3021 B) at 4.00°C, with a device for initial boundary sharpening. The optical gradient was registered by Philpot-Svensson optics. Calculations were based on the displacement of the inflection points of gradient curves and corrected to standard conditions. Relative viscosities were measured with the capillary viscometer of the Ubbelohde-Cannon type thermostated at $20° \pm 0.01°C$; the flow time of distilled water at 20°C was 295.2 sec. No corrections were made for kinetic energy of the effluents.

Hydrodynamic properties were determined in 0.2 M-Tris-HCl buffer, pH 7.3, containing 0.005 M-EDTA.

## RESULTS

*Isolation of aldolase.* The purification procedure is summarized in Table 1.

*Crude extract.* Human skeletal muscle was minced and suspended in 3 vol. (w/v) of 5 mM-Tris-HCl - 5 mM-EDTA - 4 mM-2-mercaptoethanol solution, pH 7.5. The suspension was homogenized in a blendor for 60 sec and then centrifuged at 15 000 *g* for 1 h.

*Ammonium sulphate fractionation.* The supernatant was brought to 0.54 saturation by gradual addition of solid $(NH_4)_2SO_4$ (33 g/100 ml) during 2 h. After standing for 1 h, the solution was centrifuged at 15 000 *g* for 1 h. The precipitate was discarded and the resulting supernatant was brought to 0.59 ammonium sulphate saturation by the addition of solid $(NH_4)_2SO_4$ (3.1 g/100 ml). pH was then adjusted to 7.5 with 6 N-$NH_4OH$ and the solution was allowed to stand for at least 2 h before centrifugation at 15 000 *g* for 1 h. The 0.54 - 0.59 ammonium sulphate precipitate was dissolved in 10 mM-Tris-HCl - 1 mM-EDTA - 1 mM-2-mercaptoethanol, pH 7.5, to contain 30 - 40 mg protein/ml. The solution was desalted on Sephadex G-25 equilibrated with the same buffer.

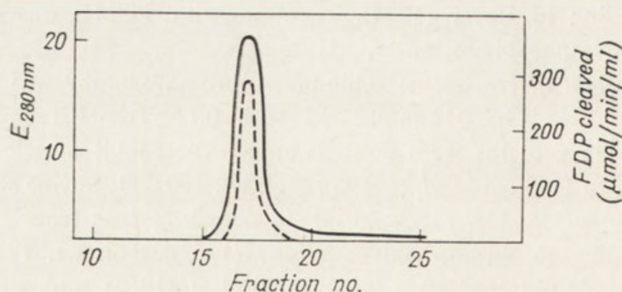


Fig. 1. Phosphocellulose chromatography of human muscle aldolase. The 0.54 - 0.59 sat. ammonium sulphate fraction obtained from the extract of human muscle (400 g) was chromatographed as described in the text, and fractions of 3 ml were collected. ——, $E_{280}$; - - -, activity.

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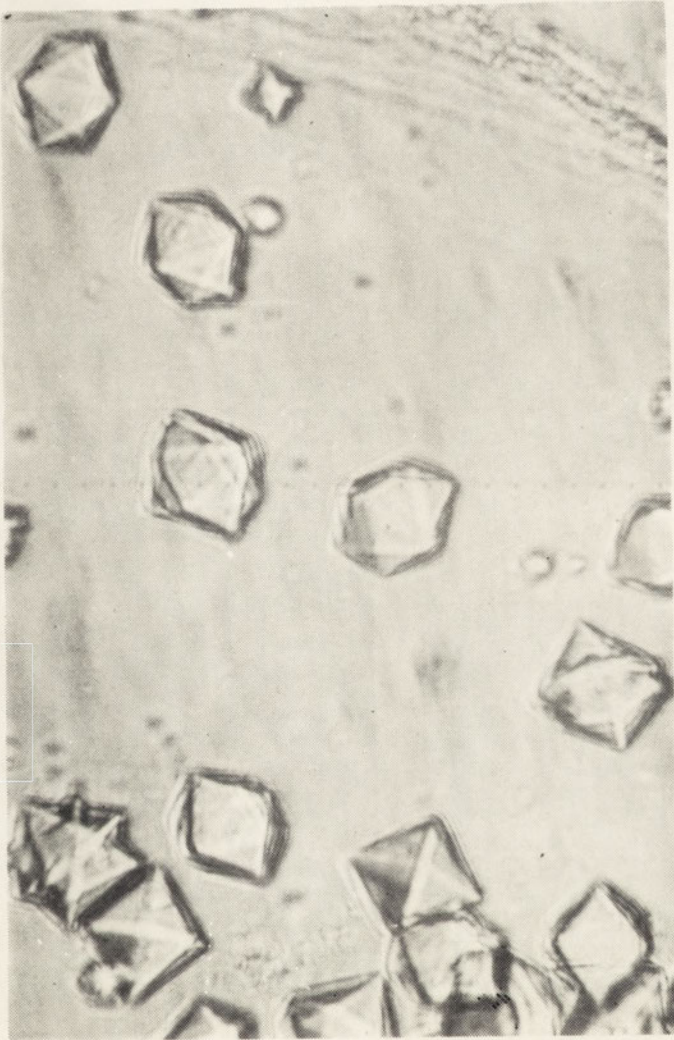

Fig. 2. Photomicrograph of crystalline human muscle aldolase. Magnification 160×.



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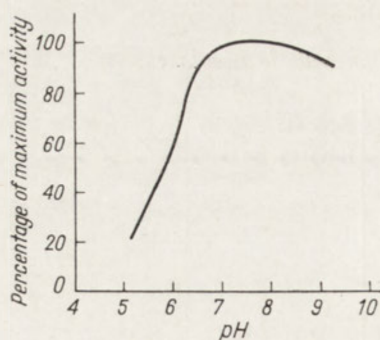


Fig. 7

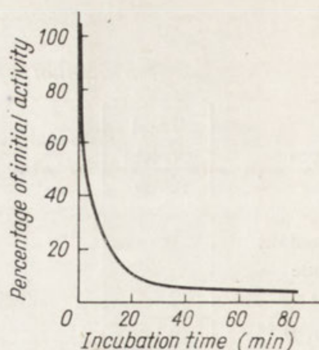


Fig. 8

Fig. 7. The effect of pH on FDP cleavage by human muscle aldolase. The activity was assayed as described in Methods except that 0.1 M-Tris-malonate buffer was used instead of Tris-HCl.

Fig. 8. The effect of carboxypeptidase A treatment on FDP cleavage by human muscle aldolase. The enzyme dissolved in 0.1 M-Tris-HCl - 1% LiCl, pH 7.5, was incubated as described under Methods. Aliquots were removed from the reaction mixture at the time intervals indicated and assayed spectrophotometrically.

*Peptide maps.* From Fig. 6 it may be seen that the peptide maps of aldolases from human and rabbit muscle are closely similar.

*Carboxy-terminal residue.* The results of hydrazinolysis as well as carboxypeptidase digestion indicate that tyrosine is the carboxy-terminal amino acid of human muscle aldolase.

*Sulphydryl groups.* Titration of human muscle aldolase with PCMB after Boyer (1954) showed the presence of 27.4 sulphydryl groups per mole of the enzyme.

*Hydrodynamic properties and molecular weight.* Mol. wt. of human muscle aldolase, estimated primarily by t.l.c., was found to be 165 000. Other methods of mol. wt. determination were subsequently used. The observed sedimentation coefficients, $s_{20,w}$, were plotted against protein concentration (Fig. 9). The linear least square

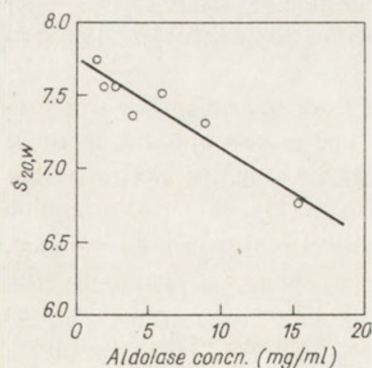


Fig. 9. Sedimentation coefficient of crystalline human muscle aldolase as a function of protein concentration at pH 7.3

equation corresponding to the straight line drawn was $s_{20,w} = s^0_{20,w} - k \times c$, where $s^0_{20,w} = 7.74$ S and $k = 0.60$. The diffusion coefficient depended only slightly on concentration, yielding at infinite dilution a value of $D^0_{20,w} = 4.51 \times 10^{-7}$ cm$^2$ sec$^{-1}$.

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The limiting viscosity number ($\eta$) was found to be 0.040 dl $g^{-1}$. The partial specific volume, $\bar{V}$, was taken to be 0.737 $g^{-1}$, the value measured by Reisler & Eisenberg (1969) for rabbit muscle aldolase. From the extrapolated sedimentation and diffusion coefficients, mol.wt. $M_{s,D}=159\,000$ was calculated.

The mol.wt. of the enzyme was also obtained by combining the sedimentation and viscosity data according to Scheraga & Mandelkern (1953). The parameter of the equation was assumed to be $2.12\times10^6$, the value for spheres and compact ellipsoidal molecular of moderate axial ratio. The limiting viscosity number precludes molecular conformation such as elongated rod or a random coil (Tanford, 1961). The mol.wt. calculated in this way was $M_{s,\eta}=155\,000$.

From the presented results it may be concluded that molecular weight of human and rabbit muscle aldolases is the same, about 160 000.

## DISCUSSION

It is commonly known that homogeneity of aldolase preparations depends to a large extent on conditions of isolation of the enzyme. This is particularly important with respect to the human muscle aldolase obtained by autopsy. Penhoet *et al.* (1969a) pointed to the possible degradation or modification of the enzyme due to digestion by proteolytic enzymes present in crude extracts. Degraded aldolase A exhibits low specific activity with FDP and low FDP:F-1-P activity ratio. The best preparations of the rabbit muscle enzyme have a specific activity with FDP about 16 - 18 i.u./mg, and the FDP:F-1-P activity ratio about 50. Since comparison of aldolases A obtained from skeletal muscles of vertebrates suggested a high degree of homology (Anderson *et al.*, 1969; Ting *et al.*, 1971), similar values could be expected for the human muscle enzyme. However, Ikehara *et al.* (1969) reported for this enzyme much lower values, i.e. specific activity close to 1.3 and the FDP:F-1-P activity ratio of 37. These divergences suggest that the Taylor's procedure used by Ikehara may not permit preparation of highly purified aldolase.

Isolation of human muscle aldolase by the method of Penhoet *et al.* (1969a) has been reported from our laboratory (Dżugaj *et al.*, 1971). At the same time a similar isolation procedure was described by Dikow *et al.* (1971). The specific activity of the preparation obtained by these authors was close to 5.5 but the FDP:F-1-P activity ratio was even lower than that found by Ikehara and amounted to 13.

The method described in the present paper yielded homogeneous preparations with high specific activity (about 16) and the FDP:F-1-P activity ratio close to 48. This high degree of purity was achieved mainly by shortening of the isolation procedure, and application of chromatography on phosphocellulose combined with precipitation of the active protein at a very narrow range of ammonium sulphate saturation.

The properties of the aldolase preparation obtained resemble closely those of the rabbit muscle enzyme. Molecular weight of both enzymes is the same, 160 000. On the basis of the same molecular weight and very similar peptide maps of both enzymes it is concluded that the structure of human muscle aldolase is tetrameric.

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This conclusion is supported by the results of hybridization experiments performed by Rudnicka and Kochman (cf. Kochman & Kwiatkowska, 1972) in which three hybrid forms were obtained. The results of polyacrylamide-gel electrophoresis of the human enzyme in 8 M-urea prove the occurrence of two types of subunits, similarly as in the rabbit muscle enzyme (Chan *et al.*, 1967). COOH-terminal group and the number of SH groups are also the same in both aldolases. Therefore it may be presumed that any existing differences between these two enzymes result from a few point mutations.

Recently human muscle aldolases of high specific activity have been obtained by two other groups of workers (Eagles & Iqbal, 1973; Allen *et al.*, 1973). The data reported on the properties of these preparations are in close agreement with our results. The low FDP:F-1-P activity ratio of the preparation of Allen *et al.* (1973) suggests, however, partial degradation of the enzyme.

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The authors thank Dr. Marian Kochman for his interest in the progress of this work and helpful discussion.

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## OTRZYMYWANIE I WŁAŚCIWOŚCI ALDOLAZY Z MIĘŚNI LUDZKICH

Streszczenie

1. Otrzymano krystaliczną aldolazę fruktozo-1,6-dwufosforanu z mięśni ludzkich. Wyniki uzyskane przy pomocy elektroforezy na żelu poliakrylamidowym, elektroforezy na polioctanie celulozy oraz w ultrawirówce analitycznej wskazują na homogenność enzymu.

2. Aktywność specyficzna względem FDP wynosi około 16 m.j./mg białka, stosunek aktywności FDP do F-1-P około 48.

3. Ciężar cząsteczkowy oznaczony na podstawie pomiarów właściwości hydrodynamicznych oraz filtracji żelowej wynosi 160 000.

4. Przy pomocy trawienia karboksypeptydazą A oraz hydrazynolizy wykazano, że C-końcowym aminokwasem jest tyrozyna. Enzym zawiera 28 grup SH i składa się z dwóch typów podjednostek różniących się ruchliwością elektroforetyczną. Mapa peptydowa ludzkiej aldolazy mięśniowej jest bardzo podobna do mapy peptydowej aldolazy z mięśni królika.

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A C T A B I O C H I M I C A P O L O N I C A

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# SOME PROPERTIES OF HIGHLY PURIFIED ARYLSULPHATASE A FROM HUMAN PLACENTA

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For the purified arylsulphatase A from human placenta tested with 2-hydroxy-5-nitrophenyl sulphate as substrate, the pH optimum was 4.9 and $K_m$ 1.42±0.04 mM. The pI of the enzyme was 4.7, and molecular weight at pH 5.0, 6.0 and 7.5, about 110 000. Phosphate, sulphide, fluoride and sulphate acted as potent inhibitors, and steroid hormones had no effect on the enzyme activity. Experiments with group-specific protein reagents pointed to the requirement of histidine, amine and -SH groups for maximum activity. Cysteine prevented inactivation of the enzyme by *p*-chloromercuribenzoate.

Arylsulphatase A (arylsulphate sulphohydrolase, EC 3.1.6.1) has been isolated from several human tissues: brain (Balasubramanian & Bachhawat, 1963; Harinath & Robins, 1971), liver (Baum *et al.*, 1958a,b; Neuwelt *et al.*, 1971), kidneys (Stinshoff, 1972), placenta (Gniot-Szulżycka *et al.*, 1967, 1970) and urine (Breslow & Sloan, 1972; Stevens *et al.*, 1973).

In the present work some properties of highly purified, stable form of the enzyme from human placenta are described and compared with those of arylsulphatase A from other sources.

## MATERIALS AND METHODS

*Chemicals.* Dipotassium 2-hydroxy-5-nitrophenyl sulphate (NCS) was prepared according to Roy (1958) and crystallized several times from glass-distilled water. Other reagents used were from the following sources: bovine serum albumin, crystalline (Sigma, St. Louis, Mo., U.S.A.); ovoalbumin, 5×cryst. (Serva, Heidelberg, G.F.R.); transferrin from human pooled plasma (Calbiochem, Los Angeles, Calif., U.S.A.); $\gamma$-globulins (Mann Research Lab., New York, U.S.A.); yeast hexokinase



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and estriol (Fluka A.G., Buchs S. G., Switzerland); caeruloplasmin from human serum, cholesterol and deoxycorticosterone (Polfa, Poland); horse myoglobin, cortisone, corticosterone, progesterone and pregnenolone (Koch-Light Lab., Colnbrook, Bucks., England); dehydroisoandrosterone (L. Light, Colnbrook, Bucks., England); estradiol (Merck, Darmstadt, G.F.R.); estrone (Loba-Chemie, Wien-Fischamend, Austria); methylotestosterone (B.D.H. Lab., Chem. Div., England); Sephadex G-200, superfine and DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden).

*Arylsulphatase A* was prepared from placentae of healthy women as described previously (Gniot-Szulżycka & Komoszyński, 1970). The 1300-fold purified stable enzyme preparation, which on agar-gel electrophoresis separated into two fractions, was used. The activity of the preparation was 40 μmol of 4-nitrocatechol/mg protein/min.

The enzyme activity was determined according to the method of Robinson *et al.* (1951). The standard reaction mixture contained 10 mM-2-hydroxy-5-nitrophenyl sulphate (NCS), 0.5 M-acetate buffer, pH 5.0, and an appropriate amount of enzyme preparation. After 10 min incubation at 37°C, the reaction was stopped by adding 3 ml of 5% NaOH solution, and the liberated 4-nitrocatechol measured at 510 nm.

*Molecular weight* was determined by thin-layer gel filtration on Sephadex G-200 superfine, suspended in the following solutions: 0.05 M-acetate buffers, pH 5.0 or 6.0; 0.05 M-Tris-HCl buffer, pH 7.5, or in the same buffers containing 0.1 M-KCl. The suspension after swelling was spread on a glass plate (29 × 40 cm) and equilibrated for 12 h in a chromatography chamber. Then the arylsulphatase A preparation, dialysed previously against the appropriate buffer solution, and reference proteins (1% solutions) were applied. The plate was developed at an angle of 30° for 5 h. The protein was detected with 1% ethanolic solution of bromophenol blue, and the enzyme activity using Whatman 3 *MM* paper soaked with 30 mM-NCS in 1.5 M-acetate buffer, pH 5.0. After 2 h at 37°C, the paper was alkalized; the red spots of liberated 4-nitrocatechol marked the position of the enzyme.

*Isoelectric point determination.* This was done by the method of Lampson & Tytell (1965) except that DEAE A-50 Sephadex instead of CM-Sephadex A-50, and 0.125 M-veronal-acetate buffer instead of 0.15 M-phosphate buffer, were used.

*Effect of inorganic salts.* Samples of the enzyme (0.4 μg of protein) were preincubated for 30 min at 25°C with various concentrations of the salts studied, then the activity was determined. Iodination and treatment with diazobenzoate were performed as described by Fraenkel-Conrat (1957).

*Effect of steroids.* To the enzyme preparation (0.6 μg of protein in 0.1 ml of water), steroid hormone solution in 1% Triton X-100 was added and the activity determined. The reaction was stopped with 3 ml of 0.5% NaOH, instead of 5% NaOH solution, to prevent turbidity of Triton X-100 which appears in strong alkaline solution.

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*Effect of group-specific protein reagents.* The enzyme preparation (2 μg of protein) in 0.24 ml of water was incubated for 1 h at 0 - 4°C with 0.24 ml of the inhibitor solution adjusted to the pH value indicated in Table 1. Then the sample was dialysed for 20 - 24 h against glass-distilled water at 4°C, made up with water to 10 ml, and in sample containing 0.2 μg of protein the activity was determined. Control samples adjusted to the appropriate pH, were dialysed under the same conditions.

*Protein determination.* This was carried out by the spectrophotometric method of Warburg & Christian using for calculation the Kalckar's equation: 1.45 $E_{280}$ − −0.74 $E_{260}$ (Layne, 1957). In some experiments the method of Lowry *et al.* (1951) was used with dry bovine serum albumin as standard.

## RESULTS AND DISCUSSION

*Molecular weight.* The molecular weight of arylsulphatase A from human placenta estimated by Sephadex G-200 thin-layer gel filtration at pH 5.0, 6.0 and 7.5 in 0.05 M-acetate or Tris-HCl buffer, with or without the addition of 0.1 M-KCl, was found to be about 110 000 (Fig. 1). This value is the same as that obtained by Sephadex G-200 gel column filtration (Gniot-Szulżycka & Komoszyński, 1970), and is closely similar to the values reported for arylsulphatase A from ox liver (Nichol & Roy, 1964), human brain (Harinath & Robins, 1971) and ox brain (Lewosz, 1971).

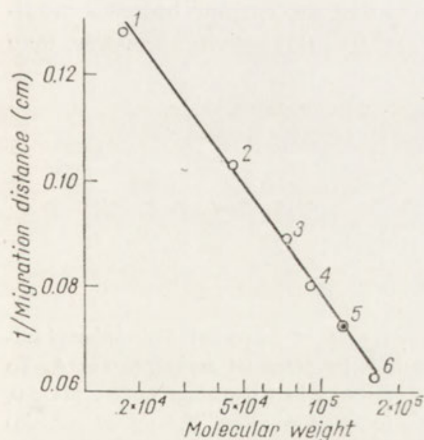


Fig. 1. Molecular weight of human placental arylsulphatase A, estimated by thin-layer gel filtration on Sephadex G-200 superfine. Conditions as described in Methods. *1*, Myoglobin; *2*, ovoalbumin; *3*, albumin; *4*, transferrin; *5*, arylsulphatase A; *6*, γ-globulins.

*Isoelectric point.* The pI value for the human placental arylsulphatase A was 4.7 (Fig. 2). This value resembles closely those found for the enzyme from human liver (Neuwelt *et al.*, 1972), kidney (Stinshoff, 1972), for the α and β forms isolated from urine (Stevens *et al.*, 1973), and for the enzyme from kangaroo liver (Roy, 1971). It differs, however, from the pI value of 3.4 found for arylsulphatase A from ox liver (Nichol & Roy, 1964).

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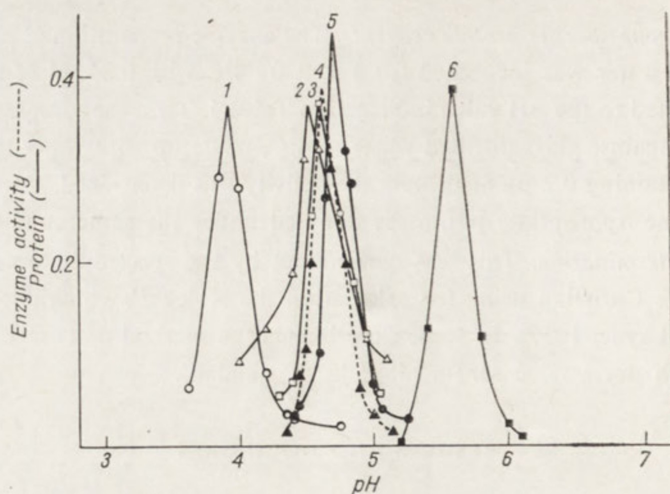


Fig. 2. Isoelectric point determination for human placental arylsulphatase A. Standard proteins (1% solutions in 0.125 M-veronal-acetate buffer, pH 6.0) and arylsulphatase A (5 μg of protein) were applied to the DEAE-Sephadex column (1×10 cm) equilibrated with the same buffer. The column was eluted with 5 ml portions of the buffer, the pH in each successive portion being lower by 0.2 pH unit. Protein ($E_{600}$) was determined by the method of Lowry *et al.* (1951), and arylsulphatase A activity at $E_{510}$. *1*, Caeruloplasmin; *2*, hexokinase; *3*, ovoalbumin; *4*, arylsulphatase A; *5*, albumin; *6*, transferrin.

*Kinetics.* The time-activity curve presented in Fig. 3 shows the anomaly typical for arylsulphatases isolated from different sources (Baum *et al.*, 1958a,b; Nichol & Roy, 1964; Stinshoff, 1972). It has been suggested that the enzyme becomes modified by the substrate which results in a loss of activity; the activity, however, may be partially restored by the reaction products.

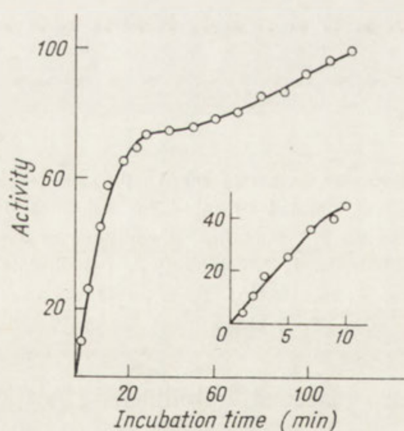


Fig. 3. Time-course of 2-hydroxy-5-nitrophenyl sulphate hydrolysis by placental arylsulphatase A. To 1 ml of 20 mM-NCS in 1 M-acetate buffer, pH 5.0, 1 ml of the enzyme solution (6.5 μg of protein) was added, the mixture incubated at 37°C, and 0.03 ml samples were withdrawn at the indicated time intervals. The activity is expressed as nmol of 4-nitrocatechol liberated/ sample.

The $K_m$ value for the hydrolysis of NCS, calculated from the Lineweaver-Burk plot (Fig. 4) was found to be 1.4±0.04 mM. It is practically the same as the $K_m$ of 1.7 reported for human liver arylsulphatase A (Neuwelt *et al.*, 1971) and 1.54

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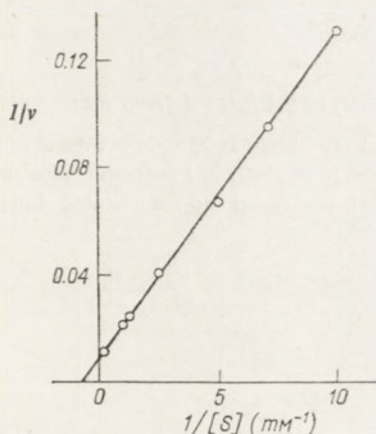
for human brain enzyme (Balasubramanian & Bachhawat, 1963), but higher than the value of 0.47 for the ox liver enzyme (Nicholls & Roy, 1971). According to Stinshoff (1972) the differences in $K_m$ values are dependent on the concentration of monovalent anions and the incubation time.



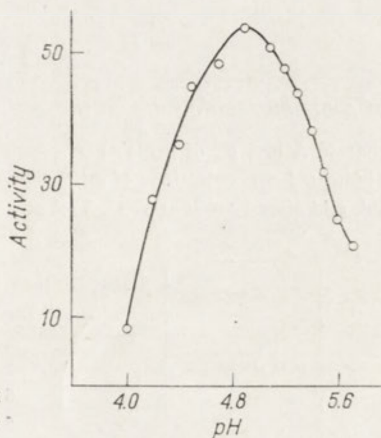




Fig. 4 Fig. 5

Fig. 4. The Lineweaver-Burk plot for 2-hydroxy-5-nitrophenyl sulphate hydrolysis by arylsulphatase A from human placenta. To the reaction mixture, 0.1 ml, which contained varying concentrations of NCS in 1.0 M-acetate buffer, pH 5.0, was added 0.1 ml of the enzyme preparation (0.58 µg of protein). The incubation time was 5 min at 37°C. *v* is expressed as nmol of liberated 4-nitrocatechol/ 5 min/sample.

Fig. 5. Effect of pH on the activity of arylsulphatase A from human placenta. The reaction mixture contained 0.1 ml of 20 mM-NCS in 1.0 M-acetate buffer, pH 4.0 - 5.8, and 0.1 ml of arylsulphatase A preparation (0.14 µg of protein). The incubation time was 10 min at 37°C. The activity is expressed as nmol of 4-nitrocatechol liberated/10 min/sample.

*Optimum pH*. Our purified preparation showed optimum activity at pH 4.8 - 5.0 (Fig. 5). The pH optimum for arylsulphatases from different sources is known to vary from 4.4 to 6.9 and it seems to depend on time of incubation and on enzyme and substrate concentration. The enzyme A from human liver (Baum *et al*., 1958a,b) after 10 min incubation exhibited two pH optima, at 4.4 and 5.2, whereas after a longer time or at higher enzyme concentration only one peak at pH 4.8 was noted. On the other hand, the purified arylsulphatase A from the same source (Neuwelt *et al*., 1971) showed two peaks at pH 4.6 and 5.0 even after a long time of incubation. Homogeneous ox liver arylsulphatase A (Nichol & Roy, 1964) and chicken brain arylsulphatase A (Farooqui & Bachhawat, 1972) showed optimum activity at pH 5.6 after a short, and at pH 5.0 after a long time of incubation. For ox brain arylsulphatase A two pH optima, at pH 5.2 and 5.8, were found after 10 min incubation (Błeszyński, 1967). The enzyme preparation from hog intestine (Szafran & Szafran, 1964) exhibited only one pH optimum at 6.0, and the enzyme from kangaroo liver, at pH 6.9 (Roy, 1971). These differences in the pH optima might point to the exi-

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stence of the same arylsulphatase A molecule in various states, or might point to the presence of different tissue- or species-dependent forms of the enzyme.

*Effect of inorganic ions.* Among the compounds tested (Table 1), phosphate, sulphide and fluoride were found to be potent inhibitors, sulphate had a moderate inhibitory effect, whereas chloride had practically no effect.

Table 1

*Effect of some inorganic salts on the activity of arylsulphatase A from human placenta*

Arylsulphatase A (0.4 μg of protein) was preincubated for 30 min at 25°C in a volume of 0.2 ml with the indicated concentrations of salts; then 0.2 ml of 20 mM-NCS in 1 M-acetate buffer, pH 5.0, was added, and after 10 min at 37°C, 3 ml of 5% NaOH was added and the liberated 4-nitrocatechol determined.

| Substance | Concentration in the preincubation mixture (M) | Residual activity (%) |
|---|---|---|
| $KH_2PO_4$ | $5\times10^{-6}$ | 98 |
| | $5\times10^{-4}$ | 51 |
| | $5\times10^{-3}$ | 7 |
| | $1\times10^{-1}$ | 0 |
| $Na_2SO_3$ | $5\times10^{-6}$ | 100 |
| | $5\times10^{-4}$ | 87 |
| | $5\times10^{-3}$ | 11 |
| | $1\times10^{-1}$ | 0 |
| NaF | $5\times10^{-6}$ | 100 |
| | $5\times10^{-4}$ | 100 |
| | $5\times10^{-3}$ | 48 |
| $Na_2SO_4$ | $5\times10^{-4}$ | 100 |
| | $5\times10^{-3}$ | 89 |
| | $1\times10^{-1}$ | 13 |
| NaCl | $5\times10^{-3}$ | 100 |
| | $1\times10^{-1}$ | 106 |

*Effect of steroids.* Cortisone, corticosterone, deoxycorticosterone, dehydroisoandrosterone, estriol, estradiol, estrone, progesterone, testosterone, methylotestosterone, pregnenolone and cholesterol, at final concentrations of 2.5, 25 and 250 mM, had no effect on the enzyme activity.

*Effect of group-specific protein reagents.* As shown in Table 2, the placental arylsulphatase A was highly sensitive to -SH group reagents, as $AgNO_3$ and PCMB. The inactivation by PCMB was prevented by cysteine. The insensitivity to iodoacetamide and cysteine excluded the presence of free -SH and S-S bridges in the active centre of the enzyme molecule. The total inhibition caused by iodination and diazobenzoate suggest the presence of histidine in the active centre. The inhibition caused by acetylation (70%), low inhibitory effect of alanine with formaldehyde (6%) and moderate inhibition in the presence of acetamide with formaldehyde (41%) point to the presence of amine group in the active centre.

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Table 2

*Effect of group-specific protein reagents on the activity of arylsulphatase A from human placenta*

The enzyme (2 µg of protein) was preincubated for 1 h at the indicated concentration of the reagent, then dialysed against water and the activity determined. For details see Methods. Mean values from 4 experiments are given.

| Reagent, at concentration | pH of the preincubation mixture | Residual activity (%) |
|---|---|---|
| $Ag(CH_3COO)$, 1 mM | 7.4 | 2 |
| PCMB, 5 mM | 7.4 | 3 |
| PCMB, 5 mM+cysteine, 60 mM | 7.4 | 98 |
| Iodoacetamide, 5 mM | 7.3 | 102 |
| Cysteine, 0.2% | 7.0 | 132 |
| Diazobenzoate, 1% | 7.0 | 0 |
| Formaline, 2%+alanine, 2% | 7.0 | 96 |
| Formaline, 2%+acetamide, 2% | 7.0 | 59 |
| Iodination, 5 mM-$I_2$ in 50 mM-KI | 5.4 | 0 |
| Acetylation | 5.6 | 30 |

On the whole, the results on the action of group-specific protein reagents are in good agreement with those reported by Meangwyn-Davies & Friedenwald (1954) for the enzyme from rabbit liver, by Błeszyński & Leźnicki (1967) for ox brain, and Jerfy & Roy (1969) for ox liver.

It should, however, be noted that the reported results concerning the inhibition of arylsulphatase A by PCMB, differ rather widely. The ox liver enzyme was insensitive to 0.1 mM-PCMB at pH 7.5 (Jerfy & Roy, 1969), whereas the enzyme isolated from ox brain (Błeszyński & Leźnicki, 1967) and human brain (Balasubramanian & Bachhawat, 1963) were inactivated at pH 7.2 by 6 mM and 0.1 mM-PCMB, respectively, and the inactivation was not prevented by cysteine or glutathione.

A closely related thiol reagent, *p*-hydroxymercuribenzoate (PHMB) was found by Neuwelt *et al.* (1972) to inhibit to a different extent urinary arylsulphatase A from healthy children (up to 94%), and from patients with the late infantile metachromatic leukodystrophy (from 43 to 78%). On the other hand, Stumpf & Austin (1971) observed resistance of the enzyme to PHMB treatment.

It should be noted that Stevens *et al.* (1973) separated arylsulphatase A from human urine into forms $\alpha$ and $\beta$; form $\alpha$ was precipitated by 1.6 M, and $\beta$ by 2.4 M-ammonium sulphate. Arylsulphatase A from human placenta was precipitated by 1.6 M-ammonium sulphate (Gniot-Szulżycka & Działoszyński, 1967), similarly as form $\alpha$.

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## WŁAŚCIWOŚCI WYSOKOOCZYSZCZONEGO PREPARATU ARYLOSULFATAZY A Z ŁOŻYSKA LUDZKIEGO

### Streszczenie

Oczyszczony preparat arylosulfatazy A z łożyska ludzkiego, badany wobec siarczanu 2-hydroksy-5-nitrofenolu jako substratu, ma optimum pH 4.9, $K_m$ 1,42±0,04 mM. Punkt izoelektryczny enzymu jest przy pH 4,7, ciężar cząsteczkowy badany w pH 5,0, 6,0 i 7,5 wynosi 110 000. Fosforany, siarczyny, fluorki i siarczany są silnymi inhibitorami enzymu. Hormony sterydowe nie mają wpływu. Doświadczenia ze związkami działającymi na specyficzne grupy białka wskazują na obecność histydyny, grupy aminowej i -SH w centrum aktywnym. Cysteina zapobiega inaktywacji enzymu przez PCMB.

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# STUDIES ON ELECTRON SPIN RESONANCE OF $Cu^{2+}$ COMPLEXES WITH NUCLEOSIDES*

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The interaction of copper with nucleosides was investigated by the method of electron spin resonance. The magnetic parameters of the $Cu^{2+}$ complexes with nucleosides were determined. Spin densities on orbitals $d_{x^2-y^2}$ and $d_{xy}$ of the central ion were calculated. It was found that the investigated compounds listed in order of decreasingly covalent nature of the copper-nucleoside bond assume the sequence: deoxycytidine = = cytidine > inosine > deoxyguanosine = guanosine > deoxyadenosine = adenosine > deoxythymidine > uridine = ribose = deoxyribose.

The ESR method, which is widely used in biology (Ingram, 1969) has also been applied in studies on the interaction of nucleic acids with metal ions (cf. the reviews by Phillips, 1966; Weser, 1968; Izatt *et al.*, 1971; Zimmer, 1971).

On the basis of ESR studies on $Cu^{2+}$ complexes with deoxynucleosides, deoxynucleotides and DNA, Ropars & Viovy (1965) described three types of copper binding with DNA: electrostatic — with phosphate groups, covalent — with phosphate groups, and "internal" — with nitrogen bases. The ability of deoxyribonucleosides to form $Cu^{2+}$ complexes decreased in the following order: deoxyguanosine> deoxycytidine>deoxyadenosine. Deoxythymidine exhibited no interaction with the copper ion. The corresponding sequence in the case of ribonucleosides was found to be: cytidine>guanosine>adenosine>thymidine>uridine=deoxyribose (Bemski *et al.*, 1971). According to Berger *et al.* (1972), nucleosides and deoxynucleosides bind copper in different ways.

The ESR method is superior to other procedures in permitting to determine directly, on the basis of the hyperfine structure of ESR lines, the delocalization

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of spin density, and thus to demonstrate the covalent nature of the bond between the central atom and ligand. The ESR spectrum of the cupric ion shows a hyperfine structure resulting from the interaction with its own nucleus ($I=3/2$). Sometimes, hyperfine splitting caused by the nuclei of the ligand atoms is also visible.

The present study was designed to calculate the distribution of spin density in the $Cu^{2+}$ complexes with some nucleic acid components, on the basis of the experimentally determined magnetic parameters of these complexes.

## MATERIALS AND METHODS

*Reagents.* Adenosine, deoxyadenosine, ribose and deoxyribose (Koch-Light, Colnbrook, Bucks, England), guanosine, inosine (British Drug Houses, Poole, Dorset, England), deoxyguanosine (Calbiochem, San Diego, Calif., U.S.A.), deoxycytidine·HCl, cytidine, uridine, deoxythymidine (Serva, Heidelberg, West Germany) and cupric sulphate of reagent grade (Office of Distribution of Chemical Reagents, Gliwice, Poland) were used. Deoxycytidine hydrochloride was converted into deoxycytidine according to Miles (1963).

*ESR measurements.* For the ESR studies, $8\times10^{-3}$ M solutions of nucleosides and pentoses in $1.6\times10^{-3}$ M-$CuSO_4$ - $2\times10^{-3}$ M-NaCl, pH 5.5 - 5.6, were used. Solid samples were obtained by freeze-drying of the aqueous solutions; the ratio of nucleoside to $Cu^{2+}$ varied from 1:1 to 10:1.

The ESR studies were carried out on an ERP-X-3 Unit (Wrocław Technical University). Measurements were taken at room temperature and at 77°K, with the use of manganous standard ($Mn^{2+}$ in MgO) and DPPH (1,1-diphenyl-2-picrylhydrazide).

*The magnetic parameters of the complexes.* Spectroscopic splitting factor $g_{\parallel}$ was obtained from the value of magnetic field $H_{res}$ corresponding to the point lying midway between lines 2 and 3 of the hyperfine structure quartet. For determination of $g_{\perp}$ the use was made of the analysis of asymmetrical curves, developed by Peter *et al.* (1962). The hyperfine interaction constant A of the copper nucleus was determined by measuring the distance between the different lines of the hyperfine structure visible on the $g_{\parallel}$ side.

## RESULTS AND DISCUSSION

ESR spectra of solid samples observed at room temperature in no case exhibited a hyperfine structure related to the hyperfine interaction of the unpaired electron with the nucleus. Only at 77°K with increasing ratio of the ligand to $Cu^{2+}$, this hyperfine structure became progressively evident; however, even at the ligand:$Cu^{2+}$ ratio = 10 only two clearly visible quartet components were observed. Typical spectra obtained are presented in Fig. 1.

A well defined hyperfine structure was observed on freezing the aqueous solutions at 77°K (Fig. 2). The parameters of the respective ESR spectra obtained for the $Cu^{2+}$ complexes with the investigated compounds at 77°K are shown in Table 1.

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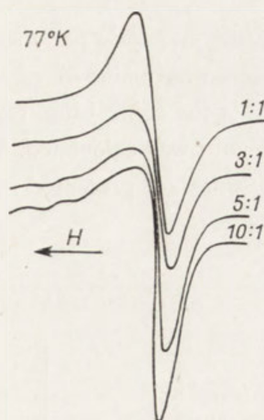


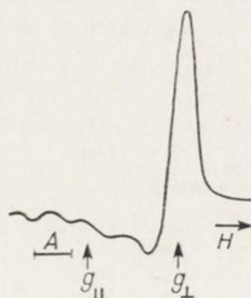


Fig. 1 Fig. 2

Fig. 1. ESR spectra of freeze-dried complexes of inosine with copper, at 77°K. Ratio of ligand to metal indicated in the Figure.

Fig. 2. ESR spectrum of frozen aqueous $8\times10^{-3}$ M solution of deoxyguanosine and $1.6\times10^{-3}$ M solution of $CuSO_4$, pH 5.6, 77°K.

Table 1

*Parameters of the ESR spectra of* $Cu^{2+}$ *complexes with nucleosides at* 77°K

| Complex | $g_{\parallel}$ | $g_{\perp}$ | $g_{aver.}$* | A (gauss) |
|---|---|---|---|---|
| Adenosine | 2.278 | 2.054 | 2.128 | 140 |
| Deoxyadenosine | 2.277 | 2.056 | 2.129 | 139 |
| Cytidine | 2.232 | 2.022 | 2.092 | 160 |
| Deoxycytidine | 2.238 | 2.022 | 2.094 | 158 |
| Guanosine | 2.275 | 2.050 | 2.125 | 143 |
| Deoxyguanosine | 2.274 | 2.048 | 2.123 | 145 |
| Deoxythymidine | 2.364 | 2.056 | 2.159 | 111 |
| Uridine | 2.362 | 2.066 | 2.165 | 119 |
| Inosine | 2.267 | 2.033 | 2.111 | 144 |
| Ribose | 2.362 | 2.066 | 2.165 | 112 |
| Deoxyribose | 2.367 | 2.068 | 2.168 | 115 |

$*g_{aver.}=\frac{1}{3}\,(g_{\parallel}+2g_{\perp})$

The unpaired spin density responsible for the electron spin resonance spectrum is partly localized on the central atom, and in part — delocalized on the ligand atoms. In the description of the ESR results obtained for the cupric ion,. use was made of the theory of molecular orbitals developed for copper complexes by Maki & McGarvey (1958) and Kivelson & Neiman (1961). Application of the theory of molecular orbitals enables interpretation of the ESR data in the case of cupric ion surrounded by oxygen or nitrogen atoms.

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On the basis of the experimentally determined magnetic parameters and optical absorption bands $\Delta_1 = E_{d_{xy}} - E_{dx^2-y^2}$, calculation was made of $\alpha^2$ and $\beta_1^2$, i.e. of the spin densities on the orbitals $d_{x^2-y^2}$ and $d_{xy}$ of the central ion, respectively. Also spin density $\alpha'^2$ of the unpaired electron on ligand was calculated. The parameters used in the calculations as well as the spin densities are presented in Table 2.

Table 2

*Spin hamiltonian constants for complexes of* $Cu^{2+}$ *with nucleosides*

| Complex | $g_{\parallel}$ — — 2.0023 | $g_{\perp}$ — — 2.0023 | $\Delta_1$ ($cm^{-1}$) | A ($cm^{-1} \times$ $\times 10^2$) | $\alpha^2$ | $\alpha'^2$ | $\beta_1^2$ |
|---|---|---|---|---|---|---|---|
| Adenosine | 0.276 | 0.052 | 14 100 | 1.320 | 0.704 | 0.394 | 0.932 |
| Deoxyadenosine | 0.275 | 0.054 | 14 300 | 1.310 | 0.701 | 0.397 | 0.942 |
| Cytidine | 0.230 | 0.020 | 14 000 | 1.507 | 0.697 | 0.401 | 0.804 |
| Deoxycytidine | 0.236 | 0.020 | 13 900 | 1.488 | 0.697 | 0.401 | 0.816 |
| Guanosine | 0.273 | 0.048 | 14 100 | 1.347 | 0.707 | 0.391 | 0.919 |
| Deoxyguanosine | 0.272 | 0.046 | 14 100 | 1.361 | 0.709 | 0.389 | 0.914 |
| Deoxythymidine | 0.362 | 0.053 | 12 500 | 1.045 | 0.715 | 0.362 | 1.000 |
| Uridine | 0.360 | 0.064 | 12 500 | 1.121 | 0.738 | 0.338 | 0.981 |
| Inosine | 0.265 | 0.031 | 13 700 | 1.356 | 0.694 | 0.404 | 0.894 |
| Ribose | 0.360 | 0.064 | 12 500 | 1.056 | 0.721 | 0.356 | 0.999 |
| Deoxyribose | 0.365 | 0.066 | 12 500 | 1.078 | 0.733 | 0.343 | 0.996 |

The values of $\alpha^2$ remain for all the complexes within the narrow range of 0.69 - 0.74, indicating that bond $\sigma$ is of a covalent nature. If $\alpha^2 = 1$, then it is assumed that the bond is of ionic character; if $\alpha^2 = 0.5$ (with the overlap integrals neglected), then binding of the central ion with the ligands may be accepted as covalent. However, since the overlap integrals are rather large, this bond cannot be unequivocally classified as covalent or ionic; it may only be assumed that the lower the value of $\alpha^2$, the more covalent the nature of the bond.

The overlap integrals in $B_{2_g}$ wave-function are small, therefore $\beta_1^2$ is a direct index of the covalency of bonds $\pi$ lying in the plane of the complex. The differences in $\beta_1^2$ value are much more pronounced than those of $\alpha^2$ (0.8 - 1.0). Consequently, $\beta_1^2$ is a better index of bond covalency than $\alpha^2$.

Parameter $g_{\parallel}$ is the most sensitive magnetic parameter, subject to the greatest changes depending on the structure of the complex. Thus, it can be considered as an index of the covalent nature of bonds. From the data presented in Table 2 it appears that the more "covalent" complexes show lower values of $g_{\parallel}$ than the "ionic" ones.

The values of: $\Delta g_{aver} = f(\Delta g_{\parallel})$ and $A = f(\Delta g_{\parallel})$ for the $Cu^{2+}$ complexes with nucleosides and pentoses are given in Fig. 3. The results indicate that the covalent nature of the copper - ligand bond is decreasing in the following sequence:

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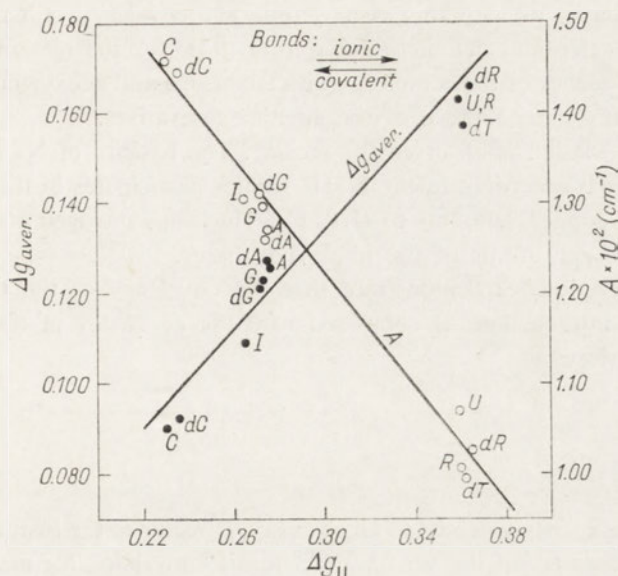


Fig. 3. Kivelson-Neiman plots of A, and $\Delta g_{aver.}$, and $\Delta g_{\parallel}$.

deoxycytidine = cytidine > inosine > deoxyguanosine = guanosine > deoxyadenosine = adenosine > deoxythymidine > uridine = ribose = deoxyribose.

These results are consistent with those of Bemski *et al.* (1971) who have obtained an analogous sequence for nucleosides.

According to the presented results, ribonucleosides and deoxyribonucleosides exhibit no differences in binding with $Cu^{2+}$. These results are in disagreement with those of Berger *et al.* (1972) who demonstrated a stereoselective binding of $Cu^{2+}$ with nucleosides and deoxynucleosides. However, in their experiments cupric acetate was used which in dimethylsulphoxide occurs in the form of a dimer. Therefore under these conditions formation of dinuclear copper aggregates was possible (Tsuchida *et al.*, 1956; Yamada *et al.*, 1957). In addition, dimethylsulphoxide as the non-aqueous solvent favours interaction of $Cu^{2+}$ with the hydroxy groups of ribose in nucleosides (Berger & Eichhorn, 1971a,b).

It is of interest to compare the differences observed in the ability of nucleosides to form $Cu^{2+}$ complexes with basicity of the nitrogen atoms participating in binding of $Cu^{2+}$. It is generally known that the more readily the donor group releases an electron pair, the stronger is the bond formed between this group and the metal ion.

$N_3$ of cytosine and $N_7$ of guanine and adenine participate in the formation of $Cu^{2+}$ complexes with nucleosides. According to Nakajima & Pullman (1958), the basicity of $N_3$ in cytosine, $N_7$ in guanine and $N_7$ in adenine are as follows: -2.25, -1.67 and -1.42 eV. One can presume therefore that binding of cytosine with copper would be the strongest, and that of adenine, the weakest. The experimental results

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obtained are consistent with this assumption. The covalency of $Cu^{2+}$ complexes with cytosine nucleosides definitely differ from those for the remaining complexes studied. The covalency of the complexes with guanosine and deoxyguanosine nucleosides only slightly exceed those of the adenine derivatives.

A distinct deviation is observed for inosine. The basicity of $N_7$ in inosine, i.e. of the atom which according to the NMR studies participates in the formation of a complex with copper, amounts to -1.19 eV. Thus, this nitrogen atom is the least basic of all nitrogen atoms of the nucleoside bases.

Still, the ESR studies demonstrate that the covalency of the $Cu^{2+}$ complex with inosine is intermediate as compared with the covalency of the cytidine and guanosine complexes.

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The author is greatly indebted to Dr. T. Luty, Director of the Institute of Physical and Organic Chemistry of the Wrocław Technical University, for making available the equipment for the ESR spectra measurements.

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## BADANIA ELEKTRONOWEGO REZONANSU PARAMAGNETYCZNEGO KOMPLEKSÓW $Cu^{2+}$ Z NUKLEOZYDAMI

Streszczenie

Badano metodą elektronowego rezonansu paramagnetycznego interakcję miedzi z nukleozydami. Wyznaczono magnetyczne parametry kompleksów miedzi z nukleozydami. Wyliczono gęstości spinowe na orbitalach $d_{x^2-y^2}$ i $d_{xy}$ jonu centralnego. Stwierdzono, że kowalencyjny charakter wiązania miedź - nukleozyd maleje w szeregu:
dezoksycytydyna = cytydyna > inozyna > dezoksyguanozyna = guanozyna > dezoksyadenozyna = adenozyna > dezoksytymidyna > urydyna = ryboza = dezoksyryboza.

Received 10 December, 1973.

*Note added in proof*: The recently obtained ESR spectra of $Cu^{2+}$ complexes with deoxyinosine, deoxyuridine and thymine riboside confirmed our results on the similarity in behaviour of riboside and deoxyriboside derivatives.

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# BIOSYNTHESIS AND METABOLISM OF CUCURBITACINS AND STEROLS AT EARLY STAGES OF DEVELOPMENT OF *CUCUMIS SATIVUS* L PLANTS

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1. Concentration profiles of sterols and cucurbitacins, and comparison of changes in total and specific radioactivity of these compounds in *C. sativus* seedlings supplied with [2-$^{14}$C]mevalonate, indicate that cucurbitacins, in contrast to sterols, are rapidly metabolized *via* glycosylation and/or degradation. 2. The results obtained are contradictory to the earlier suggestions (Enslin & Rehm, 1958, *Proc. Linn. Soc. Lond.* **169**, 230-238) that cucurbitacin C is formed by transformation of cucurbitacin B.

Two types of tetracyclic triterpenes occur in *C. sativus*: sterols (Sucrow & Reimerders, 1968) and cucurbitacins B and C (Enslin, 1954; Rehm & Wessels, 1957). Sterols are known to occur in all higher plants (Goad, 1972), and participate in formation of the lipoprotein structure (Kemp *et al.*, 1967). Cucurbitacins (Scheme 1) are typical secondary metabolites characteristic for the Cucurbitaceae plants (Rehm, 1960), in which they occur either in free form or as glycosides. Initial stages of biosynthesis are common to sterols and cucurbitacins since squalene and probably cycloartenol, formed by cyclization of squalene, serve as precursors for both types of compounds (Goad, 1972; Zander & Wigfield, 1970). It seems that biosynthetic pathways of sterols and cucurbitacins separate at the cycloartenol stage.

Biosynthesis and metabolism of sterols during germination of higher plant seeds were investigated by numerous authors (Kemp *et al.*, 1967; Kasprzyk & Fonberg-Broczek, 1967; Cowley & Evans, 1972; Bush & Grunwald, 1972; Méance & Dupéron, 1973); in some studies radioactive precursors were used (Baisted, 1962, 1969; Kasprzyk *et al.*, 1972). Much less information is available on the synthesis and metabolism of cucurbitacins and their biogenetic interrelations although a number of Cucurbitaceae plants have been investigated for the cucurbitacin content

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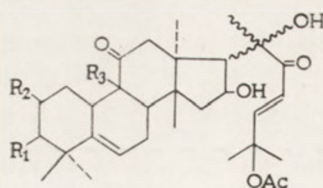


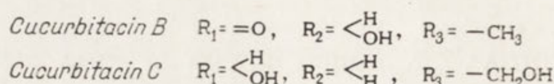


Scheme 1

(Enslin, 1954; Rehm & Wessels, 1957; Enslin & Rehm, 1958). Therefore, changes in concentration of individual cucurbitacins during germination of *C. sativus* were examined in detail. The rates of biosynthesis and degradation of these compounds were compared with those of biogenetically related sterols.

## MATERIALS AND METHODS

*Reagents.* [2-$^{14}$C](3R,S)-mevalonic acid lactone, spec. act. 10.3 mCi/mmol, was obtained from the Radiochemical Centre (Amersham, England); silica gel (Kieselgel G nach Stahl) from Merck AG (Darmstadt, G.F.R.); Rhodamine 6G and cholesterol from B.D.H. Chemicals Ltd (Poole, England); SE-30, from Carlo Erba (Milan, Italy). All solvents used for t.l.c. were freshly distilled. The crude elaterase preparation (acetone powder) was obtained from the leaves of 30-day-old *C. sativus* plants according to Enslin *et al.* (1956). The standards of cucurbitacins were kindly supplied by Dr. J. Konopa (Technical University, Gdańsk).

*Plant material. Cucumis sativus* L. var. Wisconsin plants were germinated on filter paper moistened with tap water. The seedlings were grown at stable illumination (3000 lux, 16 h per day) and temperature (day 24°C, night 16°C).

*Incorporation of* [2-$^{14}$C]*mevalonate*. Roots of 2.5-day-old seedlings were immersed in aqueous solution of [2-$^{14}$C]mevalonic acid lactone (spec. act. 10.3 mCi/mmol); 8 ml of the solution containing 25 μCi ($5.5 \times 10^7$ d.p.m.) of the radioactive precursor was used for 100 plants. After 12 h the seedlings were rinsed with water to remove mevalonate that was not taken up, and the seedlings were placed again on the moist filter paper. Samples consisting of 40 plants were taken for analysis after 0, 1/2, 1, 2, 4, 8 and 16 days after treatment.

*Isolation of sterols and cucurbitacins.* Cotyledons and embryo axes were separately homogenized in methanol. The homogenates were filtered and the residue was extracted 3 times with hot methanol. The combined extracts were evaporated to dryness and the residue was exhaustively extracted with *n*-butanol. The butanol extracts were washed with water, concentrated and fractionated into: sterols ($R_F$ 0.85), free cucurbitacins B and C ($R_F$ 0.46 and 0.40, respectively) and the glycosides ($R_F$ 0.0 - 0.2) by t.l.c. on silica gel using chloroform - ethyl acetate - formic acid (48:48:4, by vol.) (Curtis & Meade, 1971); appropriate standards were run

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simultaneously. Sterols and free cucurbitacins were eluted from silica gel with diethyl ether and purified by rechromatography on silica gel using chloroform - methanol, 95:5 (cucurbitacins B and C) or diethyl ether - chloroform - methanol, 20:10:1 (sterols) as the solvent systems. The compounds were localized under u.v. light after spraying the plates with acetone solution of Rhodamine 6G. Radiochemical purity was examined autoradiographically. The glycoside fraction was eluted from silica gel with hot methanol and then subjected to enzymic hydrolysis.

*Enzymic hydrolysis of the glycoside fraction.* The glycoside fraction dissolved in methanol (0.2 ml) was incubated with crude elaterase preparation (6 mg) in 0.1 M-acetate buffer, pH 5.2 (6 ml) for 24 h at 37°C. The reaction was stopped by heating the mixture for 5 min at 100°C. Proteins were centrifuged off and cucurbitacins extracted with diethyl ether and separated by t.l.c. as described above.

*Quantitative determination of cucurbitacins and sterols.* Cucurbitacins B and C were estimated spectrophotometrically at 229 and 231 nm, respectively. Sterols were acetylated by treatment with an excess of pyridine - acetic anhydride mixture (1:1, v/v) at room temperature for 24 h. The solvents were then removed by vacuum evaporation. A known amount of cholesterol was added as internal standard, and the mixture was assayed by gas-liquid chromatography using a Pye Unicam 104 instrument fitted with flame ionization detector (FID) and all-glass columns (283 × 0.6 cm) filled with 1% SE-30 on Chromosorb W, at 250°C.

*Radioactivity measurements.* Radioactivity was determined using a Beckman scintillation counter with an efficiency of about 85%.

## RESULTS

Changes in concentration of sterols and cucurbitacins and the rates of their synthesis were followed during growth of *C. sativus* seedlings, starting with 2.5-day-old plants up to the 19th day of growth, i.e. to the initial stage of leaf formation. Cotyledons and embryo axes were analysed separately. The content of the investigated compounds and radioactivity data are referred to fresh weight of 100 seedlings; the increase in fresh weight of whole seedlings, cotyledons and embryo axes is shown in Fig. 1.

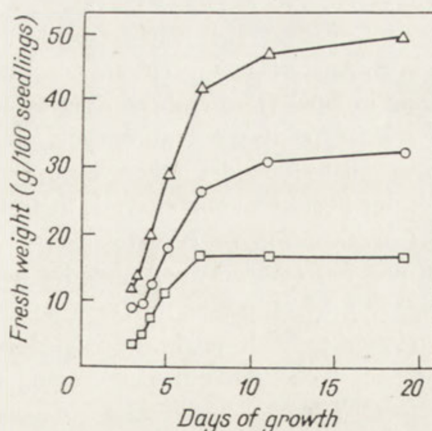


Fig. 1. Changes in fresh weight of growing *C. sativus* seedlings. (△), Whole seedlings; (○), cotyledons; (□), embryo axes.

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Data presented in Fig. 2 show that the content of sterols increased in embryo axes more rapidly than did fresh weight (Fig. 1): between the 3rd and 19th day of growth, the concentration of sterols was enhanced 134-fold, whereas the increase in fresh weight was only 4.5-fold. A similar phenomenon was observed in cotyledons between the 3rd and 11th day of growth. However, in the later period, sterol content in cotyledons decreased by about 1/3.

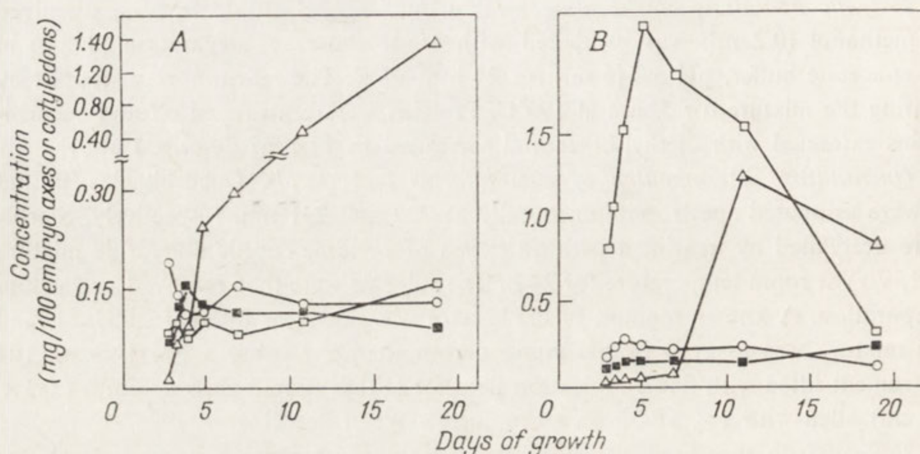


Fig. 2. Time-course of changes in concentration of sterols and cucurbitacins in *A*, embryo axes, and *B*, cotyledons of *C. sativus* seedlings. (△), Free sterols; (○), free cucurbitacin B; (□), free cucurbitacin C; (■) glycoside-bound cucurbitacin C.

Gas-liquid chromatography revealed that the sterol fraction, both in cotyledons and embryo axes, consisted of complex mixtures containing mainly 24-ethylcholesta-7,22,25-trien-3$\beta$-ol (75 - 85%) and 24-ethylcholesta-7,25-dien-3$\beta$-ol (7 - 10%). This is in agreement with the results obtained by Sucrow & Reimerders (1968) for cucumber seeds.

It has been proved that cucurbitacin C, similarly as cucurbitacin B, occurs both in embryo axes and cotyledons. This is in disagreement with the earlier reports of Enslin (1954) who detected in the embryo axes of *C. sativus* only cucurbitacin B. Analysis of u.v. spectra confirmed identity and high purity of both cucurbitacins isolated from embryo axes by t.l.c. Using this technique and a crude preparation of elaterase — an enzyme specific for cucurbitacin glycosides — we have proved that glycosides only of cucurbitacin C occur in *C. sativus* since cucurbitacin B is not liberated upon enzymic hydrolysis of the glycoside fractions.

Concentration of cucurbitacins in embryo axes was relatively low (0.1 - 0.2 mg per 100 embryo axes) and did not markedly fluctuate in the period studied (Fig. 2A), which means that concentration of these compounds decreased abruptly in relation to fresh weight. In cotyledons, the level of free cucurbitacin B and glycoside-bound cucurbitacin C decreased similarly as in growing embryo axes. The content of free cucurbitacin C in cotyledons was, on the other hand, relatively

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high and varied in the period studied: between the 3rd and 5th day its concentration increased rapidly up to 2.2 mg per 100 cotyledons, then it decreased to about 0.35 mg (Fig. 2B).

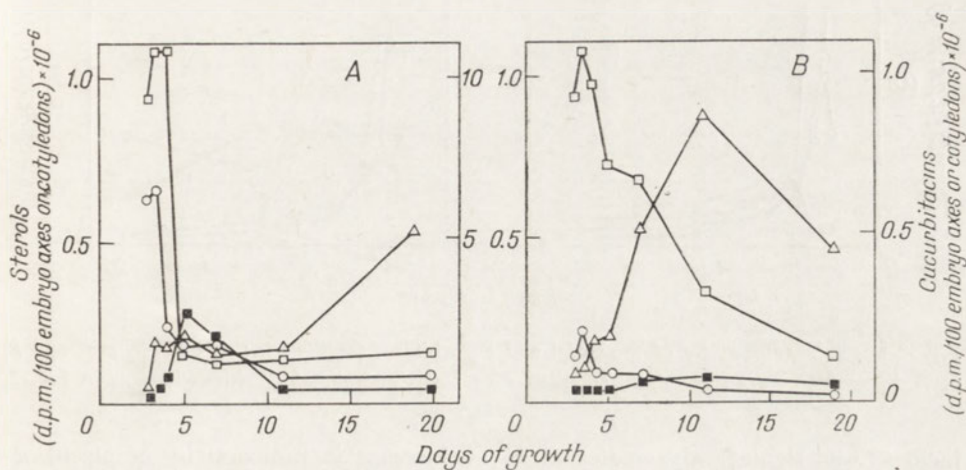


Fig. 3. Changes in total radioactivity of sterol and cucurbitacin fractions in *A*, embryo axes, and *B*, cotyledons. Incorporation of [2-$^{14}$C]mevalonate into *C. sativus* seedlings was carried out as described under Methods. Designations as in Fig. 2.

As can be seen from the results presented in Fig. 3, the rate of incorporation of [$^{14}$C]mevalonate into sterols parallelled in principle the observed increase in the total content of these compounds in the plant. This indicates relative stability of the sterols synthesized. The decrease in the incorporation of radioactive label into sterols and the drop in their total content in ageing cotyledons could be due either to formation of bound sterols such as esters and glycosides, or by transportation of free sterols to embryo axes. An immediate decrease of specific activity of the sterol fractions in embryo axes (Fig. 4A), and the decrease observed in cotyledons within the first day following treatment with [2-$^{14}$C]mevalonate (Fig. 4B) was undoubtedly caused by dilution with the rapidly synthesized non-radioactive sterols produced from storage materials or from products of photosynthesis.

The time-course of $^{14}$C incorporation into free cucurbitacins during growth of the seedlings was distinctly different (Fig. 3A,B). The highest radioactivity of cucurbitacins B and C which appeared as early as 12 h after supplying the precursor, was followed by a very rapid decrease in the radioactivity of these compounds. This proves that biosynthesis of cucurbitacins B and C was markedly faster than that of sterols, and, on the other hand, it indicates rapid transformation of cucurbitacins. This involves at least glycosylation of cucurbitacin C since a rapid decrease in radioactivity of free cucurbitacin C was associated both in embryo axes and cotyledons with a pronounced increase in radioactivity of glycoside-bound cucurbitacin C between 12 and 48 h after supply with radioactive precursor (Fig. 3A,B). However, it is noteworthy that the observed decrease in radioactivity of free cucurbi-

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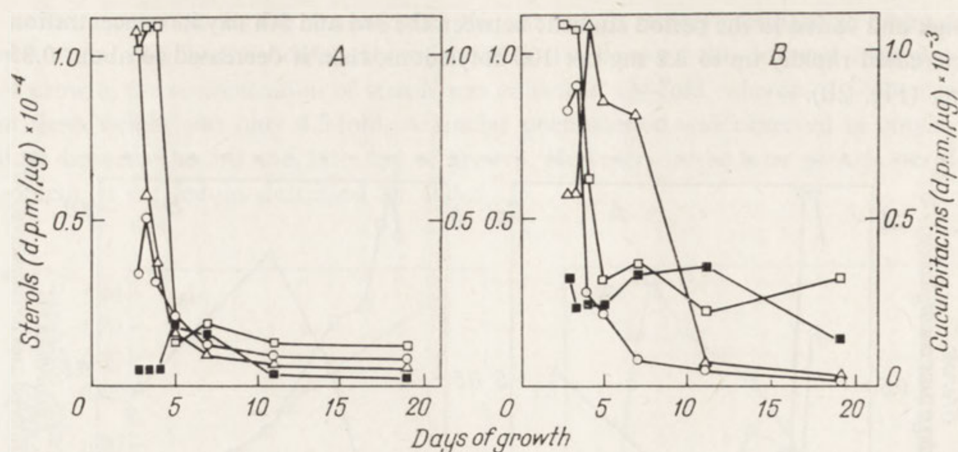


Fig. 4. Specific radioactivity of the sterol and cucurbitacin fractions in: *A*, embryo axes and *B*, cotyledons of *C. sativus* seedlings, supplied with [2-$^{14}$C]mevalonate. Designations as in Fig. 2.

tacin C was significantly greater than the increase in radioactivity of glycoside-bound cucurbitacin C. Changes in radioactivity of cucurbitacin C were very much the same as those observed for free cucurbitacin B, which, as found, was not glycosylated. This in turn indicates clearly that cucurbitacins in cotyledons and embryo axes are not only glycosylated but also undergo other fast metabolic transformations, probably degradation. This assumption is confirmed by the time-course of changes in specific radioactivity of free and glycoside-bound cucurbitacins (Fig. 4A,B).

## DISCUSSION

The results obtained indicate different behaviour of sterols and cucurbitacins in growing *C. sativus* seedlings. Rapid accumulation of free sterols both in embryo axes and cotyledons may reflect the requirement of sterols for the formation of lipoprotein membrane structures in the embryo tissues. A similar increase in the content of free sterols was observed also by Cowley & Evans (1972) in germinating seeds of *Digitalis purpurea* and by Bush & Grunwald (1972) in *Nicotiana tabacum*.

The time-course studies on labelling of free sterols with [2-$^{14}$C]mevalonate in *C. sativus* show high metabolic stability of this fraction. In contrast to sterols, cucurbitacins showed rapid turnover. The results of isotopic experiments proved that free cucurbitacins accumulated neither in the embryo axes nor in cotyledons, although they were synthesized at high rates in *C. sativus* seedlings. Rapid decrease in labelling of these compounds clearly indicates that free cucurbitacins B and C were rapidly metabolized, glycosylation of cucurbitacin C occurred, however, only to a small extent, and cucurbitacin B was not glycosylated at all.

Enslin & Rehm (1958) postulated transformation of cucurbitacin B into cucurbitacin C. The parallellity observed in the present work between [2-$^{14}$C]mevalonate

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incorporation into cucurbitacin B and C, and in particular the markedly lower specific radioactivity of cucurbitacin B than that of cucurbitacin C on withdrawal of the radioactive precursor, do not support this hypothesis.

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## BIOSYNTEZA I METABOLIZM KUKURBITACYN I STEROLI WE WCZESNYCH ETAPACH ROZWOJU ROŚLIN *CUCUMIS SATIVUS* L

### Streszczenie

Zbadano względne szybkości biosyntezy steroli i kukurbitacyn w siewkach *Cucumis sativus*, stosując [2-$^{14}$C]mewalonian jako prekursor. Porównanie zmian radioaktywności całkowitej i specyficznej badanych związków wskazuje, że kukurbitacyny — w odróżnieniu od steroli — są szybko metabolizowane, częściowo na drodze glukozylacji, częściowo ulegając degradacji. Otrzymane wyniki przemawiają przeciwko wcześniejszym sugestiom, według których kukurbitacyna C powstaje w wyniku przekształceń kukurbitacyny B.

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# VALYL-tRNA SYNTHETASE OF YELLOW LUPIN SEEDS
## PURFICATION AND SOME PROPERTIES*

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Valyl-tRNA synthetase was purified from extracts of yellow lupin seed meal by ammonium sulphate fractionation, Sephadex G-200 gel filtration, and hydrophobic chromatography on aminohexyl-Sepharose. Molecular weight of the enzyme determined by Sephadex G-200 gel filtration is 145 000. Temperature dependence of activity of valyl-tRNA synthetase suggests a different mechanism of aminoacylation reaction above and below 10°C. pH optimum is 7.5 - 8.65 in phosphate buffer and 8 - 9 in Tris-HCl buffer. Michaelis constants (in Tris-HCl buffer, pH 8.5) for tRNA, valine, ATP and $Mg^{2+}$ are $9\times10^{-7}$ M, $2\times10^{-6}$ M, $2\times10^{-5}$ M and $2\times10^{-3}$ M, respectively. The enzyme activity is very sensitive to monovalent cations. Effect of pH, buffer, salts, glycerol and substrates on thermal stability of the enzyme is described.

Despite numerous reports dealing with aminoacyl tRNA synthetases, there are still some unresolved problems in this field. Neither the mechanism of aminoacylation reaction, nor the mechanism of specific recognition of tRNA by the enzyme, have been elucidated. There is no experimental proof for the suggested explanations of the effect of salt on the activity of aminoacyl-tRNA synthetases and the degree of tRNA charging. For studying these problems, it is necessary to obtain well-characterized, homogeneous and stable preparations both of specific synthetases and tRNA.

Jakubowski & Pawełkiewicz (1973) described the purification by hydrophobic chromatography of several aminoacyl-tRNA synthetases from yellow lupin seeds. Now we present an improved procedure for purification of valyl-tRNA synthetase, and some properties of this enzyme. So far, no highly-purified and well characterized aminoacyl-tRNA synthetases have been obtained from plant material (Lea & Norris, 1972).

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## MATERIALS AND METHODS

*Materials.* tRNA was isolated from yellow lupin seeds as described by Vanderhoef *et al.* (1970); the preparation contained about 2% of $tRNA^{Val}$. Aminohexyl-Sepharose was prepared and stored as described previously (Jakubowski & Pawełkiewicz, 1973).

L-[U-$^{14}$C]Valine, spec. act. 105 mCi/mmol, obtained from UVVVR (Prague, Czechoslovakia), was diluted to 20 mCi/mmol with unlabelled valine. PPO, POPOP, Tris, L-valine, ATP disodium salt, 2-mercaptoethanol and trichloroacetic acid were from Reanal (Budapest, Hungary). Sepharose 4B, Sephadex G-200 and Blue Dextran were purchased from Pharmacia (Uppsala, Sweden). Cyanogen bromide was from Fluka AG (Buchs, Switzerland) and hexamethylenediamine from Reachim (Moscow, U.S.S.R.). Lithium chloride and rubidium chloride were from Chemapol (Prague, Czechoslovakia) and caesium chloride from B.D.H. Ltd (Poole, Dorset, England). Other reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

*Molecular weight* was determined by Sephadex G-200 gel filtration using as reference proteins: bovine serum albumin (Serva, Heidelberg, G.F.R.), calf intestine alkaline phosphatase, $\gamma$-globulin (Calbiochem, Los Angeles, Calif., U.S.A.) and myoglobin isolated from calf heart according to Bünning & Hamm (1969).

*Protein* was determined by the tannin method (Mejbaum-Katzenellenbogen, 1955) or by measuring the extinction at 280 nm (Warburg & Christian, 1941).

*Assay of valyl-tRNA synthetase activity.* Except where indicated, the reaction mixture contained in a final volume of 50 µl: 5 µmol of Tris-HCl buffer, pH 8.5, 250 nmol of $MgCl_2$, 50 nmol of ATP, 50 nmol of 2-mercaptoethanol, 0.2 - 0.3 mg of lupin tRNA preparation, 1.5 nmol [$^{14}$C]valine (20 or 105 mCi/mmol) and such an amount of the enzyme which gave the linear rate of the reaction for at least 15 min. Samples were incubated for 10 min at 37°C and then 30 µl portions were withdrawn and placed on Whatman 3 *MM* filter paper discs 2.2 cm in diameter. The discs were washed, air-dried and acid-insoluble radioactivity was counted as previously described (Jakubowski & Pawełkiewicz, 1973).

*Assay for thermal stability of the enzyme.* The standard mixture contained in a volume of 20 or 25 µl: 50 mM-potassium phosphate, pH 5.0, 10 mM-$MgCl_2$, 1 mM-2-mercaptoethanol and 3 - 4 µg of enzyme protein. The composition of the mixture varied with the experiment and is stated in the text. The samples were heated at 45.0°C up to 15 min and, after appropriate time, the remaining activity was determined after addition of 5 µmol of Tris buffer, pH 8.7, 50 nmol of ATP, 1.5 nmol of [$^{14}$C] valine (20 mCi/mmol) and 0.3 mg of tRNA in a total volume of 50 µl. As control, unheated samples were used.

## RESULTS AND DISCUSSION

### *Purification of valyl-tRNA synthetase*

In preliminary experiments it was found that both the extraction of protein from yellow lupin seeds and the extraction of leucyl- and lysyl-synthetases, was greatly affected by concentration of phosphate buffer. On the other hand, concentrat-

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ion of the buffer had practically no effect on extraction of valyl-tRNA synthetase (Fig. 1). A similar observation was made with respect to the enzymes activating isoleucine, serine, tyrosine, phenylalanine, cysteine and tryptophan (not shown in the Figure). Therefore, valyl-tRNA synthetase was extracted with 10 mM-potassium phosphate buffer containing 1 mM-2-mercaptoethanol and 10% (v/v) glycerol (buffer *A*).

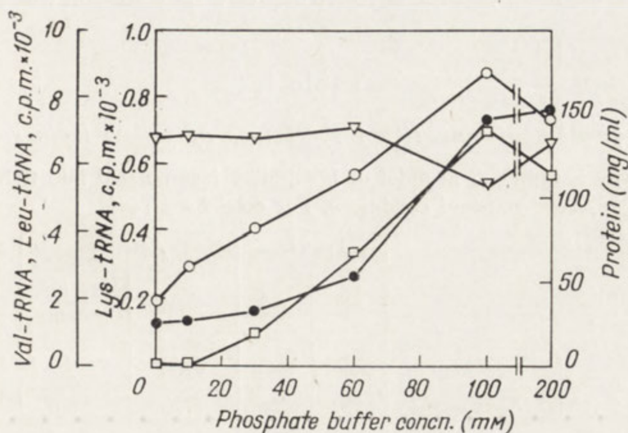


Fig. 1. Effect of phosphate buffer concentration on protein extraction from yellow lupin seeds. Portions of lupin meal, 16 g, were extracted for 30 min at 0 - 4°C with 50 ml of a solution composed of 10% glycerol, 1 mM-2-mercaptoethanol and varying concentrations of potassium phosphate buffer, pH 6.8. After centrifugation at 15 000 *g* for 30 min, in the supernatant (●) protein concentration and aminoacyl-tRNA synthetase activities for (▽) valine, (○) leucine, and (□) lysine were determined.

Extraction of valyl-tRNA synthetase from lupin seed meal and ammonium sulphate fractionation of the extract were performed as described previously (Jakubowski & Pawełkiewicz, 1973). The protein fraction precipitated at 0.35 - 0.5 $(NH_4)_2SO_4$ saturation was dissolved in a small volume of buffer *A* and applied to a Sephadex G-200 column (5 × 87 cm) equilibrated with the same buffer. Fractions of 16 ml were collected every 30 min, $E_{280}$ monitored, and activity of valyl-tRNA synthetase determined. The enzyme emerged at $V_e/V_0 = 1.45$ (void volume $V_0 = 640$ ml), fractions between 830 and 1120 ml were collected, 1 M-potassium phosphate buffer, pH 6.8, was added to 60 mM concentration, and applied to aminohexyl-Sepharose column (1.5 × 30 cm). The column was washed with 400 ml of 60 mM-potassium phosphate buffer, pH 6.8, containing 1 mM-2-mercaptoethanol and 10% (v/v) glycerol (buffer *B*) and eluted with 0 - 0.2 M-KCl concentration gradient in buffer *B* (300 ml). The valyl-tRNA synthetase activity emerged at 0.1 M-KCl. To the pooled active fractions ammonium sulphate was added to 0.7 saturation. The precipitated protein was collected by centrifugation, dissolved in a small volume of buffer *A*, and dialysed against the same buffer; then glycerol was added to 50% concentration and the preparation was stored at -20°C. Alternatively, the eluate was directly dialysed against buffer *A* containing 50% of glycerol. Both preparations

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were stable for several months. The purified preparation on polyacrylamide-gel electrophoresis, carried out according to Weber & Osborn (1969), separated into several protein bands.

The results of enzyme purification, presented in Table 1, show that a 90-fold purification was achieved in relation to the crude extract. However, taking into account the selective extraction of only a part of soluble protein by 10 mM-phosphate buffer, it may be assumed that the effective degree of purification was at least fivefold higher.

Table 1

*Purification of valyl-tRNA synthetase of yellow lupin seeds*

The enzyme activity is expressed in nmol of [$^{14}$C]valine incorporated into tRNA/10 min under standard conditions. For details see Text.

| | Protein | | Activity | | Purification | Yield (%) |
|---|---|---|---|---|---|---|
| | mg | ml | total | per mg protein | | |
| Crude extract | 4600 | 260 | 9200 | 2 | 1 | 100 |
| Ppt. at 0.35 - 0.5 $(NH_4)_2SO_4$ concn. | 1170 | 26 | 7720 | 6.6 | 3.3 | 85 |
| Sephadex G-200 eluate | 290 | 290 | 5800 | 20 | 10 | 63 |
| Sepharose-$NH(CH_2)_6NH_2$ eluate | 14.6 | 165 | 2628 | 180 | 90 | 28 |

Due to application of hydrophobic chromatography on aminohexyl-Sepharose (cf. Jakubowski & Pawełkiewicz, 1973), it proved possible to obtain a preparation of valyl-tRNA synthetase with a specific activity of 180 nmol/mg of protein/10 min at 37°C, which is the highest value so far reported for an aminoacyl-tRNA synthetase of plant origin. The activity of our preparation was sixfold higher than that of valyl-tRNA synthetase from rat liver (Yoshida *et al.*, 1969), and 20 times lower than that of the highly purified yeast enzyme (Lagerkvist & Waldenström, 1967; von der Haar, 1973).

In some cases, about 10%, the obtained enzyme preparations had a tenfold lower activity and differed in their dependence on pH and kind of buffer from the usually obtained preparations, but had the same molecular weight. Lower activity of the enzyme preparation is difficult to explain. The possibility that it could be due to differences in storage conditions of lupin seeds or their water content can be ruled out as we obtained different enzyme preparations with the seeds of the same batch. It should be noted that Lagerkvist & Waldenström (1967) also observed low activity in about 30% of the yeast enzyme preparations.

*Properties of valyl-tRNA synthetase*

*Michaelis constants* for ATP, L-valine, $Mg^{2+}$ and $tRNA^{Val}$ in the acylation reaction are presented in Table 2. The obtained results are in close agreement with those reported for other aminoacyl-tRNA synthetases (Bartman, 1969).

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Table 2

*Michaelis constants for the substrates of valyl-tRNA synthetase at pH 8.5*

In each experiment, other substrates were at saturating concentrations, as described in Methods.

| Substrate | $K_m$ (M) |
|---|---|
| Valine | $2\times10^{-6}$ |
| ATP | $2\times10^{-5}$ |
| $Mg^{2+}$ | $2\times10^{-3}$ |
| $tRNA^{Val}$ | $9\times10^{-7}$ |

*Molecular weight* of the valyl-tRNA synthetase determined by Sephadex G-200 gel filtration was found to be 145 000 daltons (Fig. 2). This value is somewhat higher than that of the enzyme from *E. coli*, 110 000 (Yaniv & Gros, 1969) and yeast, 112 000 (Lagerkvist & Waldenström, 1967; Rymo *et al.*, 1972). Since the enzymes of mol. wt. about 150 000 daltons are often composed of subunits (Loftfield, 1972) it is probable that lupin valyl-tRNA synthetase has a quarternary structure.

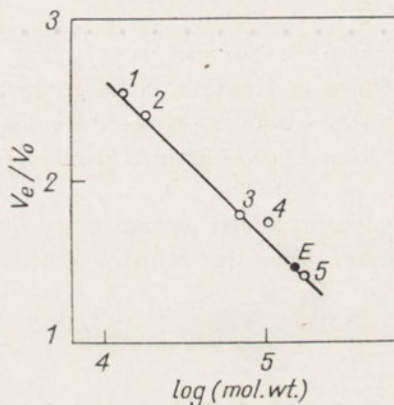


Fig. 2. Determination of molecular weight of valyl-tRNA synthetase from lupin seeds by Sephadex G-200 gel filtration. The column (2.5×80 cm of the gel) was run in buffer *A* containing 0.1 M-NaCl at a flow rate of 15 ml/h. The column was calibrated with 2 ml (5 mg/ml) samples of Blue Dextran (mol.wt. $2\times10^6$), and: *1*, cytochrome *c* ($12.7\times10^3$); *2*, myoglobin ($18\times10^3$); *3*, bovine serum albumin ($67\times10^3$); *4*, calf intestine alkaline phosphatase ($1.0\times10^5$); and *5*, γ-globulin ($1.6\times10^5$). *E*, lupin valyl-tRNA synthetase.

*Effect of temperature*. The optimum temperature for the enzyme activity was 37°C (Fig. 3), similarly as it was found for many other synthetases from yeasts and bacteria (Hirschfield & Bloemers, 1969; DeLorenzo & Ames, 1970; Christopher *et al.*, 1971; Beikirch *et al.*, 1972; Chirikjian *et al.*, 1973).

The temperature dependence of the enzyme activity plotted in Arrhenius coordinates (Fig. 4) suggests different reaction mechanisms with different standard enthalpy changes below and above 10°C (5.25 kcal/mol and 1.2 kcal/mol, respectively). The same results obtained with lysyl-tRNA synthetase from *E. coli* were interpreted by Marshall & Zamecnik (1970) as changes in enzyme conformation at the transition temperature.

*Effect of 2-mercaptoethanol and glycerol*. 2-Mercaptoethanol was found to have no effect on the activity of valyl-tRNA synthetase at concentration up to 100 mM; at higher concentration an inhibitory effect was observed. 1 M-2-Mercaptoethanol

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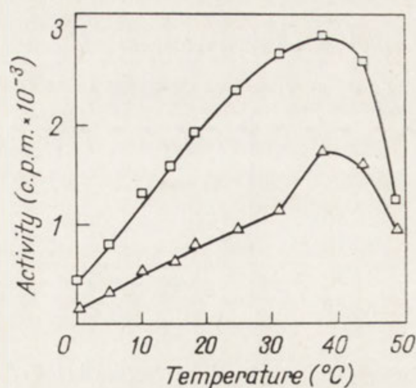


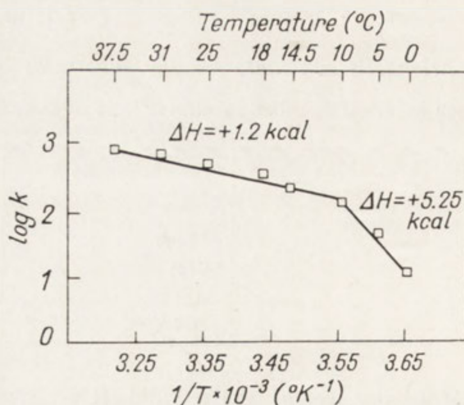




Fig. 3

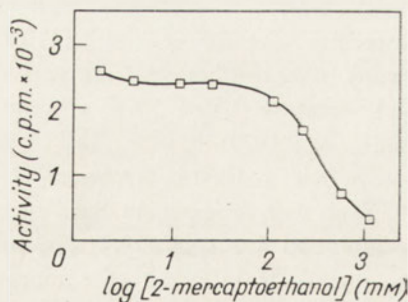
Fig. 4

Fig. 3. Effect of temperature on lupin valyl-tRNA synthetase activity. The samples were incubated for (△), 5 min and (□), 15 min at the indicated temperature. [$^{14}$C]Valine of spec. act. 105 mCi/mmol, was used. Other experimental conditions were as described in Material and Methods.

Fig. 4. Arrhenius plot of valyl-tRNA synthetase activity. Conditions as described for Fig. 3. The samples were incubated at the indicated temperature for 5 min.

inhibited the enzyme in 90% (Fig. 5). The effect of 2-mercaptoethanol can be explained as blocking of SH groups essential for enzyme activity. Only two other synthetases were reported to be inhibited by 2-mercaptoethanol: lysyl-tRNA synthetase from *E. coli* (Stern & Peterkofsky, 1969) and valine enzyme from *E. coli* (Burkard *et al.*, 1970).

Glycerol added to the incubation mixture inhibited the enzyme activity; the inhibition increased with glycerol concentration (Table 3).



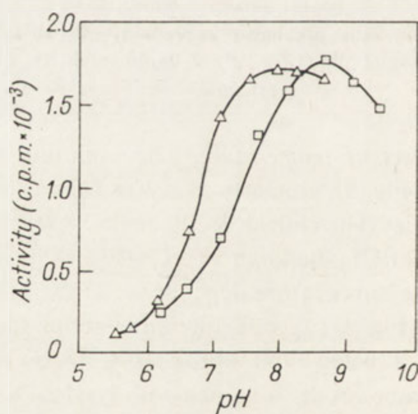




Fig. 5

Fig. 6

Fig. 5. Effect of 2-mercaptoethanol on valyl-tRNA synthetase activity. The standard reaction mixture containing varying amounts of 2-mercaptoethanol, was used.

Fig. 6. Effect of pH and kind of buffer on valyl-tRNA synthetase activity. The incubation mixture contained (△), 0.05 M-potassium phosphate buffer, or (□), 0.1 M-Tris-HCl buffer, of appropriate pH.

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Table 3

*Effect of glycerol on valyl-tRNA synthetase activity*

Activity of the enzyme was determined under standard conditions in the presence of the indicated amounts of glycerol.

| Glycerol concentration (%) | Activity (%) |
|---|---|
| 0 (control) | 100 |
| 5 | 97 |
| 10 | 80 |
| 15 | 82 |
| 20 | 69 |

*Effect of pH and kind of buffer.* The pH optimum for valyl-tRNA synthetase was 7.5 - 8.65 in 0.05 M-phosphate buffer, and pH 8 - 9 in 0.01 M-Tris-HCl buffer (Fig. 6). At pH 7 the activity of our preparation in phosphate buffer was twice as high as in Tris buffer; at lower and higher pH values the difference decreased and eventually disappeared.

There are numerous reports concerning the effect of the kind of buffer on the activity of aminoacyl-tRNA synthetases. For example, Joseph & Muench (1971) observed that tryptophanyl-tRNA synthetase from *E. coli* was equally active and showed the same pH-dependence in 0.1 M-Tris-HCl or potassium cacodylate buffers but was inhibited by 0.1 M-potassium phosphate buffer. The activity of tyrosyl-tRNA synthetase from *E. coli* at pH 7.6 was sixfold higher in 0.15 M-Tris-HCl buffer than in 0.15 M-cacodylate buffer (Beikirch *et al.*, 1972). Yeast leucyl-tRNA synthetase was more active in 0.1 M-potassium cacodylate or HEPES buffers than in 0.1 M-Tris-HCl buffer at the same pH values (Chirikjian *et al.*, 1973).

It seems that the marked influence of the kind of buffer on the aminoacylation reaction may be due to the specific effect of different buffer ions on macromolecular components of the reaction mixture. One may expect here different interactions with positively charged ions, e.g. of the type of ammonium ion (Tris), and negatively charged ones like phosphate or cacodylate. Another important factor is ionic strength of the systems investigated. It is interesting to note that in many cases pH optima of aminoacylation reactions are shifted, in Tris buffer, towards higher pH values in comparison to those in phosphate or cacodylate buffers.

*Effect of monovalent cations.* Lithium, sodium, potassium, rubidium, caesium and ammonium chlorides at concentrations up to 50 mM slightly stimulated valyl-tRNA synthetase (Fig. 7). Higher concentrations inhibited the enzyme, total inhibition being observed with 0.3 M-sodium, potassium, rubidium or caesium ions, with 0.4 M-ammonium, and at above 0.5 M concentration in the case of lithium. We cannot offer any explanation for this comparatively small effect of lithium; however, it should be noted that similar results were reported by Smith (1969) for leucyl-tRNA synthetase from *E. coli*.

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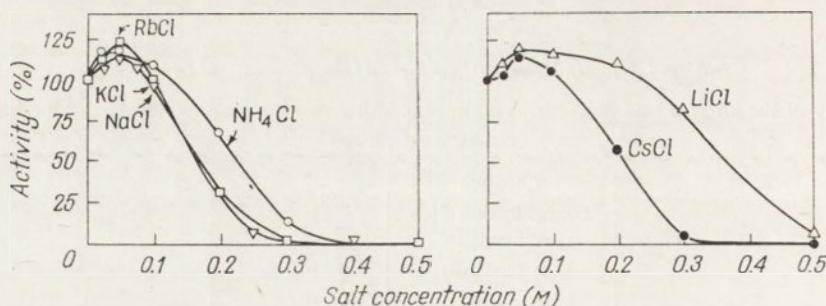


Fig. 7. Effect of monovalent cations on valyl-tRNA synthetase activity. The incubation mixtures contained the appropriate monovalent cation. Activity of the enzyme was determined as described in Materials and Methods.

The effect of low and high KCl concentration on the time-course of aminoacylation is presented in Fig. 8; 0.2 M-KCl inhibited both the initial velocity and the maximal activity.

Our results are in accordance with the overall picture of the action of monovalent cations on aminoacyl-tRNA synthetase activities: at low concentration the cations are without effect or slightly stimulate, whereas at high concentration (above 0.1 M) they inhibit the activity. The effect of salts on aminoacylation described here for valyl-tRNA synthetase of lupin, was also observed for leucyl-tRNA

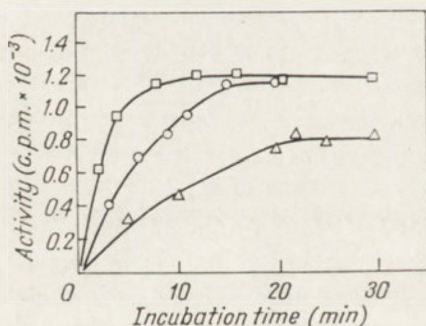


Fig. 8. Effect of (□), 0.05 M- and (△), 0.2 M-KCl on valyl-tRNA synthetase activity. (○), Control, without KCl.

synthetase (Smith, 1969; Rouget & Chapeville, 1971) and valyl-tRNA synthetase (Bonnet & Ebel, 1972) of *E. coli*, as well as of valyl-, phenylalanyl-, lysyl- and leucyl-tRNA synthetases from rat liver (Sein & Bećarević, 1971). However, there is no explanation of the observed phenomenon. It was suggested that monovalent cations influence the conformation of the synthetase (Svensson, 1967) or tRNA (Rouget & Chapeville, 1971; Sein & Bećarević, 1971). With only partially purified synthetase preparations, the monovalent cations could activate the tRNA-degrading enzymes (Sein & Bećarević, 1971).

Bonnet & Ebel (1972) suggested that inhibition of aminoacylation by salts is the result of changes in the equilibrium between aminoacylation and enzymic and non-enzymic deacylation reactions. So far, however, there is no experimental proof for this suggestion. In our opinion, monovalent cations can change the equilibrium

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of aminoacylation reaction by acting on conformation of tRNA and/or synthetase. This suggestion is supported by the observed changes in the affinity of the synthetases for tRNA, caused by monovalent salts (Santi *et al.*, 1971; Yarus, 1972; Pingoud *et al.*, 1973).

*Stability of the enzyme at* 45°C

*Effect of pH and salt concentration.* Thermal stability of the enzyme was the highest at pH 6.6, and decreased more sharply at lower than at higher pH values (Fig. 9). On addition of NaCl up to 0.1 M concentration, thermal stability of the enzyme at the optimum or at higher pH values progressively increased. Similarly, with increasing potassium phosphate concentration the enzyme became more stable at pH 6.2 and 7.5, but not at pH 5.5 (Fig. 10). Nearly identical curves were recently obtained by Norris & Fowden (1973) for asparaginyl-, lysyl- and prolyl-tRNA synthetases of *Phaseolus aureus* seeds. Other aminoacyl-tRNA synthetases

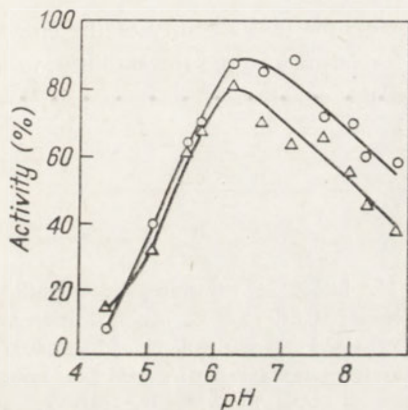


Fig. 9

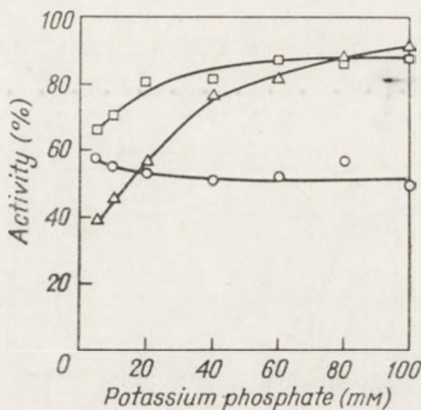


Fig. 10

Fig. 9. pH and NaCl dependence of valyl-tRNA synthetase stability at 45°C. The mixture, 20 μl, containing 0.05 M-potassium phosphate buffer of appropriate pH, (△), without or (○), with 0.1 M-NaCl was incubated for 5 min at 45°C, then the enzymic activity was determined at 37°C.

Fig. 10. Effect of potassium phosphate buffer concentration on valyl-tRNA synthetase stability at 45°C. The mixture, 25 μl, was composed as described in Materials and Methods, except that the pH of the potassium phosphate buffer was: (○), pH 5.5; (□), pH 6.2; and (△), pH 7.5. After 5 min at 45°C, the activity was determined.

so far reported show quite different pH stability curves. Lanks *et al.* (1971) reported that the stability of phenylalanyl tRNA-synthetase from rat liver at 37°C was practically pH-independent in the range of pH 6 - 8 in phosphate buffer. In the presence of salt, stability of the enzyme increased at pH 7.5 and decreased at pH 6.5. On the other hand, stability of glutamyl-tRNA synthetase of *E. coli* increased with pH over the range 6.0 - 8.0 (Lapointe & Söll, 1972).

*Effect of glycerol.* Glycerol protected the enzyme against thermal inactivation:

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at a glycerol concentration of 40%, the enzyme retained about 80% of the initial activity (Table 4).

Table 4

*Effect of glycerol on thermal stability of valyl-tRNA synthetase at pH 7.5*

After 5 min of incubation at 45°C, with or without the addition of the indicated amounts of glycerol, the activity was measured at 37°C under standard assay conditions.

| Glycerol concentration (%) | Residual activity (%) |
|---|---|
| 0 | 30 |
| 10 | 43 |
| 20 | 62 |
| 30 | 68 |
| 40 | 80 |

*Effect of substrates.* The effect of substrates on thermal stability of the enzyme is shown in Fig. 11. ATP alone and together with valine lowered the stability, valine alone had no effect, whereas tRNA protected the enzyme.

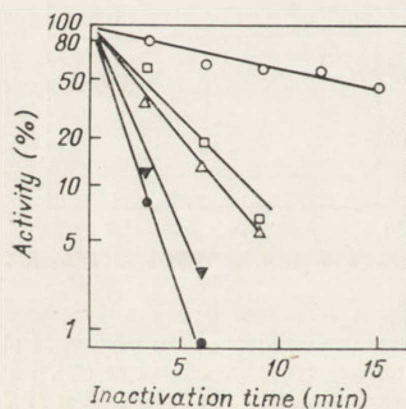


Fig. 11. Effect of substrates on valyl-tRNA synthetase stability at 45°C. The incubation mixture contained in a total volume of 20 µl: 0.05 M-potassium phosphate buffer, pH 5.0, 1 mM-2-mercaptoethanol, 7 mM-$MgCl_2$, enzyme, and appropriate substrate: (□), 1 mM-[$^{14}$C]valine; (●), 20 mM-ATP; (▼), 1 mM-[$^{14}$C]valine and 20 mM-ATP; (○), 0.1 mg of tRNA; (△), control samples. After heating for the indicated time at 45°C, the enzyme activity was determined.

The effect of tRNA is an indirect proof of tRNA-enzyme complex formation. The protection by tRNA has been observed for arginyl-, phenylalanyl- and valyl-tRNA synthetases of *E. coli* (Mitra *et al.*, 1970; Kosakowski & Böck, 1971; Yaniv & Gros, 1969, respectively), for arginyl-tRNA synthetase from rat liver (Ikegami & Griffin, 1969), isoleucyl-tRNA synthetases of *Bacillus stearothermophilus* (Charlier & Grosjean, 1972) and yellow lupin seeds (Kędzierski & Pawełkiewicz, 1970, 1971). Makman & Cantoni (1966) found no effect of yeast $tRNA^{Ser}$ on stabilization of yeast seryl-tRNA synthetase but in our laboratory (Jakubowski, unpublished) a system was developed in which this enzyme was effectively protected by yeast tRNA against heat inactivation.

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Lack of protection of lupin valyl-tRNA synthetase by valine and ATP is in contrast with the results of Norris & Fowden (1973) who reported protection of all 18 aminoacyl-tRNA synthetases from *Phaseolus aureus* by ATP and the appropriate amino acid.

The increased rate of inactivation of valyl-tRNA synthetase from yellow lupin in the presence of ATP suggests conformational changes in synthetase molecule induced by ATP. Two other synthetases, threonyl-tRNA of *E. coli* (Paetz & Nass, 1973) and phenylalanyl-tRNA of rat liver (Tscherne *et al.*, 1973), were also inactivated by ATP. In most cases, however, ATP protected aminoacyl-tRNA synthetases against thermal inactivation (Makman & Cantoni, 1966; Yaniv & Gros, 1969; Mitra *et al.*, 1970; Berry & Grunberg-Manago, 1970; Kosakowski & Böck, 1971; Charlier & Grosjean, 1972).

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## SYNTETAZA WALILO-tRNA Z NASION ŁUBINU ŻÓŁTEGO

### OCZYSZCZANIE I NIEKTÓRE WŁASNOŚCI

Streszczenie

Oczyszczono syntetazę walilo-tRNA z nasion łubinu żółtego stosując frakcjonowanie siarczanem amonu, sączenie żelowe na Sephadex G-200 i chromatografię hydrofobową na aminoheksylo-Sepharose. Ciężar cząsteczkowy enzymu oznaczony metodą sączenia żelowego na Sephadex G-200 wynosi 145 000. Krzywa zależności aktywności syntetazy walinowej od temperatury sugeruje różne mechanizmy reakcji aminoacylacji powyżej i poniżej 10°C. Optimum pH dla syntetazy walinowej wynosi 7,5 - 8,65 w buforze fosforanowym i 8 - 9 w buforze Tris. Stałe Michaelisa dla $tRNA^{Val}$, waliny, ATP i $Mg^{2+}$ wynoszą odpowiednio: $9\times10^{-7}$ M, $2\times10^{-6}$ M, $2\times10^{-5}$ M, $2\times10^{-3}$ M, (pH 8,5, bufor Tris). Sole metali jednowartościowych znacznie hamują aktywność enzymu. Opisano również wpływ pH, stężenia buforu, soli, glicerolu i substratów na stabilność termiczną syntetazy walinowej.

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# SOLUBILIZATION OF COLLAGEN FIBRILS BY HUMAN LEUCOCYTE COLLAGENASE ACTIVATED BY RHEUMATOID SYNOVIAL FLUID

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Evidence is presented that free leucocyte collagenase has limited capacity to degrade isoluble collagen fibrils. However, the addition of rheumatoid synovial fluid to the crude enzyme preparation markedly increases fibril lysis. The enzyme activity toward polymeric human skin collagen is inhibited by ethylene diaminetetraacetate, whereas diisopropylfluorophosphate and normal serum inhibit this activity only partially. Possible role of leucocyte collagenase in degradation of insoluble, mature collagen is discussed.

Recent reports indicate that highly cross-linked, insoluble collagen is less susceptible to the action of tissue collagenases than the newly synthesized, soluble collagen (Leibovich & Weiss, 1971; Harris & Farrel, 1972; Robertson & Miller, 1972). This was also found with human granulocyte collagenase (Lazarus *et al.* 1968a,b). Therefore it could be assumed that *in vivo* the minute amount of collagenase in granulocytes would have little, if any, biological significance. On the other hand, Kruze & Wojtecka (1972) have shown that in the presence of rheumatoid synovial fluid the collagenase activity of a crude preparation of human leucocyte collagenase increases severalfold; this would suggest that activation of collagenase may be of importance for collagen degradation in the inflammatory rheumatoid diseases. It was therefore of interest to compare the effect of activated and non-activated enzyme on collagen fibrils of different degree of polymerization.

## MATERIALS AND METHODS

*Reconstituted collagen fibrils* were prepared from acid-soluble calf skin collagen purified according to Kang *et al.* (1966) and from pig articular cartilage collagen isolated by the EDTA procedure of Robertson & Miller (1972) and purified accor-

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ding to Miller *et al.* (1967). A 0.2% collagen solution in 0.05% acetic acid was dialysed overnight against 0.05 M-Tris-HCl buffer, pH 7.5, containing 0.005 M-$CaCl_2$ and 0.2 M-NaCl. The dialysed solution was then centrifuged for 1 h at 10 000 *g*. The supernatant, divided into suitable portions, was polymerized at 37°C for time intervals ranging from 5 h to 10 weeks. During polymerization collagen samples were covered with a small volume of toluene to prevent bacterial growth. At a given time collagen gels were removed from the incubator and resuspended in the assay medium used for solubilization or collagenolytic tests.

*Insoluble, polymeric human skin collagen* was obtained from human dermis according to the procedure of Schofield *et al.* (1971). Human skin was obtained *post mortem* from an 82-year-old woman having no record of any connective tissue disease. Nativity of substrate fibrils was checked by estimating their susceptibility to the action of non-specific proteases. Usually, a 18 h incubation of collagen fibrils with trypsin (10:1, w/w) resulted in less than 10% digestion of the substrate.

*Crude human leucocyte collagenase* was prepared, purified and the activation of crude enzyme by rheumatoid synovial fluid was performed as described previously (Kruze & Wojtecka, 1972; Wojtecka-Łukasik & Dancewicz, 1974).

*Collagenolytic activity* was assayed in a system containing collagen fibrils suspended in a medium composed of 0.05 M-Tris-HCl buffer, pH 7.5, 0.005 M-$CaCl_2$ and 0.2 M-NaCl. The suspension (total volume 2 ml) was incubated for 18 h at 37°C with an activator and/or inhibitor at concentrations indicated. The undissolved collagen fibrils were filtered off and the content of solubilized collagen was estimated from the content of hydroxyproline in the supernatant.

*Hydroxyproline content* was determined according to Stegemann & Stalder (1967).

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

*Special reagents*: Diisopropylfluorophosphate (DFP), Tris, trypsin (Koch-Light, Colnbrook, Bucks, England); hydroxy-L-proline, pepsin 3×crystallized (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.); crystallized bovine serum albumin (Fluka A. G., Buchs, Switzerland); methyl cellosolve (Bio-Rad, Richmond, Calif., U.S.A.); guanidine hydrochloride, pure (V/O Sojuzchimexport, U.S.S.R.). All the other reagents were Polish commercial products of analytical grade.

## RESULTS

The data presented in Figure 1 illustrate degradation of insoluble human skin collagen fibrils by non-activated crude extractable leucocyte collagenase, and by the same preparation activated by rheumatoid synovial fluid. The collagenolytic activity was linearly dependent on enzyme concentration up to 3 mg of crude activated or non-activated enzyme. At higher concentrations the curve assumed the shape of a typical saturation curve (Fig. 1A).

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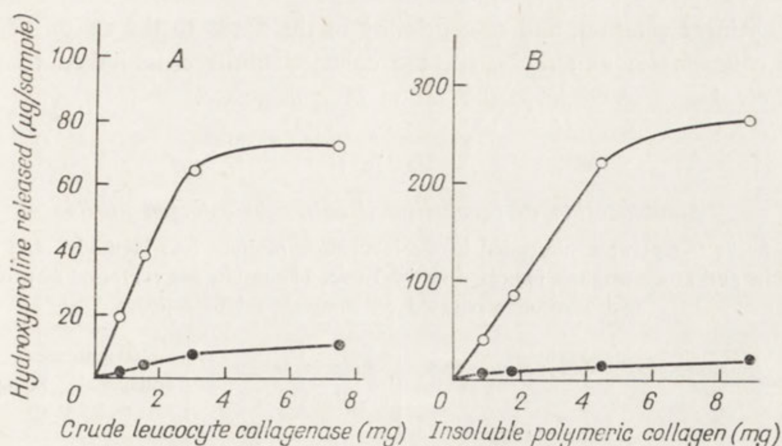


Fig. 1. Degradation of insoluble human skin collagen fibrils by crude leucocyte collagenase as a function of: *A*, Enzyme protein content, and *B*, concentration of collagen. In *A*, the reaction mixture contained 1.7 mg of insoluble polymeric collagen fibrils (210 μg of hydroxyproline) and the indicated amount of crude enzyme protein. In *B*, the reaction mixture contained 3 mg of crude enzyme protein and the indicated amount of insoluble polymeric collagen (12.3% of hydroxyproline). The activity of collagenase ●, non-activated and ○, activated by rheumatoid synovial fluid, is expressed in μg of hydroxyproline released on incubation of collagen for 18 h at 37°C. In both experiments, for activation of collagenase the rheumatoid synovial fluid was added at the protein concentration ratio of 1:1.

A typical saturation curve was also obtained when the enzymic activity of activated collagenase was plotted *versus* substrate concentration (Fig. 1B). The free non-activated collagenases showed very little activity toward insoluble collagen.

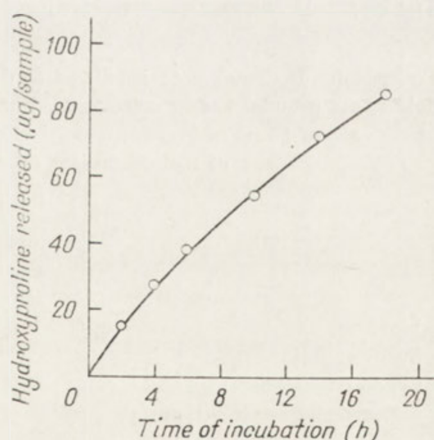


Fig. 2. Degradation of insoluble human skin collagen fibrils by activated crude leucocyte collagenase as a function of time. The reaction mixture contained 3 mg of leucocyte collagenase protein, 4.5 mg of insoluble human skin collagen (553 μg of hydroxyproline) and 3 mg of rheumatoid synovial fluid protein.

Degradation of human skin polymeric collagen by the activated collagenase was almost linear up to 18 h of incubation (Fig. 2). Data given in Table 1 illustrate close correlation between stability of reconstituted collagen fibrils in dilute acetic

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acid and Ringer solution, and susceptibility of the fibrils to the action of partially purified collagenase; as anticipated, the collagen fibrils cross-linked to a higher extent were less susceptible to the action of collagenase.

Table 1

*Solubilization of reconstituted calf skin collagen fibrils*

Collagen fibrils (1 mg) were suspended in the incubation medium (see Methods), and incubated for the time and at temperature indicated in the Table. The results are expressed as percentage of hydroxyproline released on complete solubilization.

| Incubation time | 0.2 M-acetic acid, 24 h, 4°C | Ringers solution, 10 min, 65°C | 40 × purified collagenase, 80 µg, 18 h, 37°C |
|---|---|---|---|
| 5 hours | 100 | 100 | 25.3 |
| 1 week | 100 | 100 | 22.0 |
| 2 weeks | 95 | 100 | 21.8 |
| 3 weeks | 35 | 98 | 13.3 |
| 4 weeks | 5 | 95 | 10.4 |
| 6 weeks | 3 | 59 | 6.7 |
| 8 weeks | 1 | 13 | 5.8 |
| 10 weeks | 0 | 0 | 5.0 |

This observation is supported by the results obtained with reconstituted fibrils from cartilage collagen and fibrils of insoluble human polymeric collagen used as substrates for the collagenase (Table 2). The 40-fold purified enzyme was used in

Table 2

*The effect of rheumatoid synovial fluid on degradation of collagen fibrils by leucocyte collagenase*

Collagen fibrils (2 mg) were incubated as described in Methods for 18 h at 37°C with synovial fluid (3 mg protein) and/or collagenase. Either crude or 40-fold purified enzyme preparation was used (3 mg or 80 µg of protein, respectively). Degradation of collagen is expressed as percentage of hydroxyproline released on complete solubilization.

| Addition | Reconstituted collagen fibrils of | | | Insoluble polymeric human skin collagen |
|---|---|---|---|---|
| | cartilage,* aged 48 h | calf skin, aged | | |
| | | 5 h | 10 weeks | |
| None | 13.6 | 3.5 | 4.0 | 0 |
| Synovial fluid | 20.8 | 4.0 | 5.2 | 0.8 |
| Crude collagenase | 44.5 | 13.1 | 6.2 | 3.8 |
| Crude collagenase + synovial fluid | 84.5 | 98.0 | 64.0 | 33.0 |
| Purified collagenase | 41.5 | 31.8 | 6.5 | 4.8 |
| Purified collagenase + synovial fluid | — | 31.2 | 6.0 | 4.4 |

* In experiments with cartilage collagen, 8 mg of the crude enzyme protein was added.

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the amount equivalent to the collagenase content in the crude preparation (as calculated from the protein content). The results obtained indicate that synovial fluid significantly increased lysis of collagen fibrils by the crude collagenase preparation but had no effect on the action of purified collagenase. The latter enzyme was more effective toward soluble, freshly reconstituted calf skin collagen fibrils. It was also shown that crude collagenases split more readily the cartilage collagen fibrils than those of calf skin collagen.

Table 3

*Effect of EDTA, DFP and blood serum on the collagenolytic activity of crude leucocyte collagenase activated by rheumatoid synovial fluid*

The reaction mixture consisted of 1.7 mg of insoluble polymeric human skin collagen (containing 210 μg of hydroxyproline), 3 mg of crude collagenase protein, 3 mg of the rheumatoid synovial fluid protein and the indicated inhibitors.

| Addition | Solubilized collagen (μg of hydroxyproline released) | Inhibition (%) |
|---|---|---|
| None | 71.0 | — |
| Synovial fluid omitted | 4.0 | — |
| EDTA, 10 mM | 3.0 | 96.0 |
| DFP, 1 mM | 41.0 | 42.0 |
| Blood serum (1:10 dilution) | 34.0 | 52.0 |

The results presented in Table 3 show that EDTA inhibited completely the activity of rheumatoid synovial fluid-activated crude collagenase toward insoluble human skin collagen fibrils, whereas DFP and 10% blood serum, only in 40 - 50%.

## DISCUSSION

Freshly reconstituted collagen fibrils which redissolve readily are also susceptible to the action of purified leucocyte collagenase. This finding is at variance with the observation of Lazarus *et al.* (1972) who found that purified granulocyte collagenase was less active toward collagen fibrils than a crude preparation. A different technique of preparation and purification of collagenase employed in our studies (Kruze & Wojtecka, 1972) may bear on these differences.

Decreased susceptibility of reconstituted collagen fibrils to the action of collagenase is observed upon their aging. This effect can be ascribed to the increased number of stable intermolecular cross-links formed (Deshmukh & Nimni, 1969) upon ageing. The sharp drop in solubilization of fibrils after the third week of ageing suggests that a definite number of cross-links is needed to render fibrils inaccessible to collagenase. The native, polymeric collagen, which is highly cross-linked, is even less susceptible to the action of synovial collagenase (Leibovich & Weiss, 1971) or purified leucocyte collagenase, as shown in the present work.

Limited action of tissue collagenase on polymeric collagen *in vitro* poses the question how such highly-crossed collagen is degraded *in vivo*. Leibovich & Weiss

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(1971) suggested that *in vivo* an unknown enzymic system existed which degraded collagen fibrils to monomeric form, susceptible to collagenase action. This supposition is strengthened by our results demonstrating an almost tenfold increase in digestibility of polymeric collagen by crude enzyme preparation (leucocyte homogenate) upon activation with rheumatoid synovial fluid. The synovial fluid alone or supplemented with purified collagenase had only limited action on insoluble collagen.

The results on inhibition of the activated crude collagenase offer further support for this hypothesis. DFP, which does not inhibit any known collagenase (Gross, 1970), counteracted activation of crude leucocyte collagenase by 42%. A similar effect was found with serum, which according to Lazarus *et al.* (1972) does not inhibit granulocyte collagenase, but which in our experience (Wojtecka-Łukasik & Dancewicz, 1974) inhibited partially the leucocyte collagenase. Thus, it may be supposed that the enhanced lysis of insoluble fibrils in the activated system is not due to activation of collagenase but to the presence or activation of an enzyme sensitive to DFP and specific toward insoluble, polymeric collagen.

We can only visualize this unknown enzyme as the first one in a collagenolytic system present in leucocytes. Its action is followed by that of collagenase and gelatin-specific protease (Sopata & Dancewicz, 1974) in a stepwise degradation of mature, insoluble collagen *in vivo*.

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## DEGRADACJA WŁÓKIEN KOLAGENOWYCH PRZEZ KOLAGENAZĘ LEUKOCYTÓW LUDZKICH AKTYWOWANĄ PŁYNEM STAWOWYM

Streszczenie

Wolna kolagenaza leukocytów krwi ludzkiej wykazuje ograniczoną zdolność degradowania nierozpuszczalnych włókien kolagenowych. Jednakże dodanie płynu stawowego, pobranego od pacjentów z gośćcem przewlekłym postępującym, do surowego preparatu enzymu znacznie zwiększa rozpuszczalność włókien. Aktywność kolagenolityczna uaktywnionej płynem stawowym kolagenazy leukocytów ludzkich hamowana była całkowicie przez EDTA oraz częściowo przez DFP i surowicę ludzką.

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# METHYLATION OF DNA IN HUMAN NORMAL AND LEUKAEMIC WHITE CELLS

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1. Human peripheral normal and leukaemic white cells were incubated with L-[*methyl*-$^{14}$C]methionine, and 5-methylcytosine was found to be practically the only radioactive base present in DNA of these cells. 2. Methylation of DNA was low in normal white cells and in those from patients with chronic lymphocytic leukaemia, and relatively high in cases of chronic myelogenic leukaemia. 3. The content of 5-methylcytosine in DNA of normal and the studied types of leukaemic human white cells varied somewhat but was within the range reported for various tissues of other mammals.

5-Methylcytosine (5-MC) is known to be a minor base of all animal DNA studied, and the data on DNA methylation suggest that the degree of 5-methylcytosine formation is species- and tissue-dependent (Kappler, 1971; Vanyushin *et al.*, 1970, 1973). Human normal and leukaemic white cells offer an opportunity to compare methylation of DNA in cells originating from two distinct cell lines of the same organism, at different stages of their development and different phases of the cell cycle. This material seemed of interest all the more in that Silber *et al.* (1966) reported methylation of DNA at different rates, and Desai *et al.* (1971) a different content of 5-MC in leucocytes from normal subjects and patients with leukaemia.

## MATERIALS AND METHODS

*Isolation of white cells.* Normal lymphocytes and granulocytes were separated from blood of healthy donors by stepwise sedimentation with gelatin (Malec *et al.*, 1971), the purity of lymphocyte preparations being 80 - 90% of mononuclears, and of granulocyte preparations, 90 - 98% of polymorphonuclears. Leukaemic white cells were obtained from blood of patients with chronic lymphocytic or chronic myelogenic leukaemia, during haematological relapse, at the time the patients were



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not receiving antileukaemic therapy. The white blood cell count exceeded 50 000/μl. The cells were separated by sedimentation with gelatin (Malec *et al.*, 1973).

*Labelling of DNA with* [$^{14}$C]*methyl groups.* The isolated white cells were incubated in a final volume of 100 ml as a free suspension (10 000 cells/μl) in Hanks-Simms medium (Cameron, 1950) enriched with 5% of normal human serum and 3 mg% of heparin, with the addition of 1 μCi/ml of L-[*methyl*-$^{14}$C]methionine at 37°C for 2 h. After incubation the cells were washed twice with ice-cold saline and DNA was isolated. The mean amount of the obtained DNA measured after hydrolysis was about 30 $E_{260}$ units.

*DNA isolation, purification and hydrolysis.* DNA was isolated from white cells as described by Kay (1964) and purified by the procedure of Marmur (1961). The remaining RNA was removed from the preparation by alkaline hydrolysis in 0.3 M-KOH for 1 h at 37°C (Burdon & Adams, 1969). After cooling, 1.2 M-$HClO_4$ was added, the precipitated DNA collected by centrifugation, washed twice with 0.2 M-$HClO_4$, and hydrolysed to its component bases with 70% $HClO_4$ on a boiling water bath. For chromatography, the hydrolysate was prepared as described by Kappler (1971).

*Separation of DNA bases* was performed by paper chromatography by the descending technique on Whatman no. 1 paper with a solvent system composed of propan-2-ol - 12 M-HCl - water (85:22:18, by vol.) as described by Wyatt (1951). When necessary, the eluted spots were rechromatographed with methanol - 12 M-HCl - water (7:2:1, by vol.) according to Desai *et al.* (1971). The bases were located under ultraviolet light and identified on the basis of $R_F$ values. In the 0.1 M-HCl eluate, the purity of individual bases and their content were estimated by u.v. absorption properties as described by Bendich (1957). The amount of 5-MC was determined after rechromatography according to Wyatt (1951).

*Distribution of radioactivity on the chromatogram.* To the labelled DNA hydrolysate carrier 5-methylcytosine was added and the radioactivity was determined in 1-cm strips in the Packard Tri Carb liquid scintillation counter in PPO and POPOP solution in toluene.

*Incorporation of* [$^{14}$C]*methyl groups into 5-methylcytosine of DNA.* The 5-MC spot on the chromatogram of labelled DNA hydrolysate (with the carrier 5-MC added) was cut out and the radioactivity counted directly as above. In the 0.1 M-HCl eluate of the corresponding spot, the amount of cytosine was estimated by u.v. measurement.

*Chemicals.* Adenine, guanine, cytosine and thymine used as standards were obtained from Reanal (Budapest, Hungary). 5-MC was kindly supplied by Prof. Dr. D. Shugar. Radioactive L-[*methyl*-$^{14}$C]methionine (spec. act. 46 mCi/mmol) was obtained from the Radiochemical Centre (Amersham, England).

## RESULTS AND DISCUSSION

*Methylation of DNA in various types of white cells.* Silber *et al.* (1966) reported on different rates of incorporation of [$^{14}$C]methyl groups into DNA of normal

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(mixed) peripheral white cells and leukaemic cells. However, in their experiments the radioactivity was measured in hot trichloroacetic acid extract of acid-insoluble DNA-protein pellet, which, as pointed out by Sneider & Potter (1969), cannot be considered as an adequate method for estimation of the incorporation of [$^{14}$C]methyl groups into DNA.

It was the aim of the present work to identify the product(s) which incorporated the radioactivity of [$^{14}$C]methyl group in the DNA of white cells, and to compare the extent of DNA methylation in normal and leukaemic white cells.

In DNA from normal and chronic lymphocytic leukaemia lymphocytes as well as in normal granulocytes, only a single radioactive base, namely 5-MC, was found after 2 h of incubation in the medium containing L-[*methyl*-$^{14}$C]methionine (Fig. 1). In leucocytes from patients with chronic myelogenic leukaemia a small amount (about 5%) of radioactivity appeared also in thymine, presumably by conversion of the methionine methyl group to one-carbon intermediate incorporated into thymine which would be utilized for the *de novo* DNA synthesis (Evans & Evans, 1970). The traces of radioactivity found in guanine (up to 1%) corresponded to a contamination which disappeared after rechromatography. Rechromatography of 5-MC did not affect its specific activity.

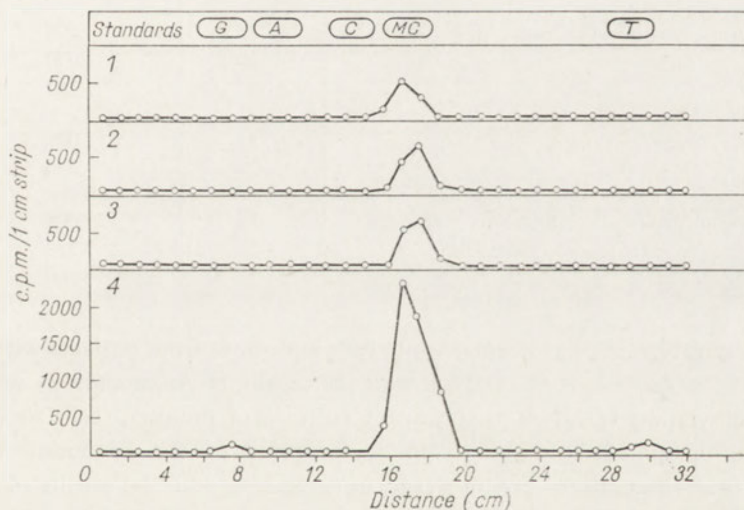


Fig. 1. Distribution of radioactivity on the chromatograms of DNA hydrolysates from various types of white cells, incubated with [*methyl*-$^{14}$C]methionine. After development, the sheet was dried and cut into strips 1 cm wide. *1*, Normal lymphocytes; *2*, chronic lymphocytic leukaemia white cells; *3*, normal granulocytes; *4*, chronic myelogenic leukaemia white cells. The diagram above the radioactivity profiles shows the location of standard bases developed at the same time.

The results of determinations of radioactivity incorporated into 5-MC of the DNA isolated from the investigated types of white cells, are presented in Table 1. Similarly to Silber *et al.* (1966), we observed a relatively high methylation of DNA in cells of patients with chronic myelogenic leukaemia, and a small but reproducible

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$^{14}$C-methylation in normal granulocytes and lymphocytes, as well as in lymphocytes from patients with chronic lymphocytic leukaemia. The significant radioactivity of 5-MC in chronic myelogenic leukaemia is also in good agreement with the findings of numerous authors (Sneider & Potter, 1969; Adams, 1971; Rubery & Newton, 1973), suggesting that the methylation proceeds efficiently only in the presence of concomitant formation of new DNA. On the other hand, the observation that some methylation of DNA occurs also in the cells which are unable

Table 1

*Incorporation of radioactivity into 5-methylcytosine of DNA of normal and leukaemic white cells*

5-MC was isolated from DNA hydrolysate by paper chromatography. The radioactivity is expressed as counts/min/μmol of cytosine present in the hydrolysate.

| Material | Subject no. | Radioactivity (c.p.m./μmol cytosine) |
|---|---|---|
| Normal lymphocytes | 1 | 984 |
| | 2 | 762 |
| Chronic lymphocytic leukaemia white cells | 1 | 904 |
| | 2 | 1 070 |
| | 3 | 1 202 |
| Normal granulocytes | 1 | 1 021 |
| | 2 | 1 230 |
| Chronic myelogenic leukaemia white cells | 1 | 25 008 |
| | 2 | 9 283 |
| | 3 | 14 560 |

to synthesize DNA, such as normal white cells and those from patients with chronic lymphocytic leukaemia, is at variance with the results of Adams (1971) who observed no methylation of DNA in stationary cultures of mouse L cells or in phytohaemagglutinin-stimulated horse lymphocytes prior to the beginning of DNA synthesis. Our observations are, however, in agreement with the results of Evans & Evans (1970) on microplasmidia *Physarum polycephalum* and of Adams & Hogarth (1973) on isolated nuclei of mouse fibroblasts, suggesting that DNA methylation is not rapidly completed but may continue for several hours after synthesis of DNA is complete.

*The content of 5-MC in DNA from white cells.* According to the data reported by Vanyushin *et al.* (1970, 1973), the amount of 5-MC in DNA from tissues of several mammals (mouse, rat, rabbit, pig, sheep and bull) is in the range of 0.90 - 1.40 mol%, the differences between particular tissues of the same animal being about 10 - 20%. In the light of those data the 0.4% content of 5-MC reported by Desai *et al.* (1971) for human (mixed) normal peripheral white cells seems to be

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Table 2

*Base composition of DNA isolated from various types of white cells*

Values are expressed as mol%.

| Material | Subject no. | A | T | G | C | A+T/ G+C |
|---|---|---|---|---|---|---|
| Normal lymphocytes | 1 | 26.5 | 30.3 | 21.2 | 22.0 | 1.31 |
| | 2 | 26.8 | 30.5 | 20.4 | 22.3 | 1.34 |
| Chronic lymphocytic leukaemia white cells | 1 | 26.7 | 29.7 | 21.3 | 22.2 | 1.30 |
| | 2 | 27.1 | 30.1 | 21.7 | 22.8 | 1.29 |
| | 3 | 27.3 | 30.2 | 20.5 | 22.0 | 1.35 |
| Normal granulocytes | 1 | 26.3 | 30.4 | 21.1 | 22.2 | 1.31 |
| | 2 | 27.2 | 29.9 | 21.1 | 21.8 | 1.33 |
| Chronic myelogenic leukaemia white cells | 1 | 27.0 | 30.5 | 20.4 | 22.1 | 1.35 |
| | 2 | 26.7 | 29.8 | 20.8 | 22.7 | 1.30 |
| | 3 | 26.9 | 29.6 | 21.5 | 22.0 | 1.30 |
| exacerbated (90% of blasts) | 4 | 27.1 | 29.7 | 20.7 | 22.5 | 1.31 |
| Acute leukaemia white cells | 1 | 26.8 | 29.9 | 20.6 | 22.4 | 1.32 |

Table 3

*5-Methylcytosine content in DNA of various types of white cells*

Mean values ±S.D. are given.

| Material | Number of subjects | 5-MC content (mol%) |
|---|---|---|
| Normal lymphocytes | 3 | 0.75±0.06 |
| Chronic lymphocytic leukaemia white cells | 6 | 0.73±0.03 |
| Normal granulocytes | 3 | 0.93±0.03 |
| Chronic myelogenic leukaemia white cells | 6 | 1.03±0.04 |
| Chronic myelogenic leukaemia, exacerbated (90% of blasts) | 1 | 1.05 |
| Acute leukaemia white cells | 1 | 1.04 |
| Spleen (Gaucher disease) | 1 | 0.77 |

very low in comparison with other mammalian tissues, and the values of 2.4 - 2.9% content of 5-MC in human leukaemic lymphoblasts, extremely high.

The composition of four main bases in DNA isolated from various types of normal and leukaemic white cells was closely similar (Table 2), and resembled the values reported by Desai *et al.* (1971). However, in contrast to the results of these authors, the 5-MC content found by us in all types of white cells studied (Table 3) were within the range of values reported for tissues of other mammals. The compa-

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rison of the values for various types of white cell seem to suggest moreover that, similarly as in various tissues of other mammals, the 5-MC content in peripheral white cells is a tissue-specific property, which finds the reflection in the differences of 5-MC content between lymphocytes (both normal and from patients with chronic lymphocytic leukaemia) and normal granulocytes. The somewhat higher value of 5-MC content found in leucocytes from patients with chronic myelogenic leukaemia in comparison with normal granulocytes might be connected with their immaturity or/and the different stage of DNA-synthesizing activity. This latter supposition could be considered in the light of Rubery's & Newton's (1973) data who have observed different extent of methylation at different rates of DNA synthesis in normal and tumour virus-transformed BHK21 cells in tissue culture. 5-MC content in human spleen was similar to that in peripheral lymphocytes.

The presented results give further evidence for the variability of the DNA methylation process in different types of cell and different cellular stages of the same organism and tissue.

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## METYLOWANIE DNA W LUDZKICH KRWINKACH BIAŁYCH PRAWIDŁOWYCH I BIAŁACZKOWYCH


### Streszczenie

1. Krwinki białe prawidłowe i białaczkowe inkubowano z L-[*metyl*-$^{14}$C]metioniną. Wykazano, że praktycznie jedyną radioaktywną zasadą w DNA tych komórek była 5-metylocytozyna.

2. Szybkość metylacji DNA w krwinkach białych osób zdrowych i chorych na przewlekłą białaczkę limfatyczną była niska; natomiast szybkość metylacji DNA w krwinkach białych osób chorych na przewlekłą białaczkę szpikową była stosunkowo wysoka.

3. W badanych typach ludzkich krwinek białych zawartość 5-metylocytozyny w DNA wykazywała pewne różnice, mieściła się jednak w granicach wartości stwierdzonych w różnych tkankach innych ssaków.


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# HISTONE CONSERVATION IN NORMAL AND HERITABLY DAMAGED MURINE LEUKAEMIC LYMPHOBLASTS L5178Y-S*

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Using prelabelling with [$^{14}$C]leucine it was found that no turnover of total histone or histone fractions *f1*, *f2a1*, *f2a2* and *f2b*+*f3*, occurred *in vitro* either in a fast growing cell population of murine leukaemic lymphoblasts L5178Y-S or in a slowly growing subline isolated from an X-irradiated L5178Y-S culture.

From the studies carried out in numerous laboratories an image emerges of histones as key factors which determine and maintain chromatin structure at the time beginning immediately after completion of DNA replication (Weintraub, 1972). For this reason knowledge of histone metabolism is essential for understanding of the molecular mechanisms of chromatin function. Several years ago Gronow & Todd (1969) suggested that growth disturbances observed in irradiated cell populations could be caused by imbalance between DNA and histone syntheses. Therefore, it seemed that studies on histone turnover in a cell population damaged by irradiation may provide interesting observations on the formation and stability of histone molecules.

The aim of this work was to examine histone turnover in a fast growing cell population of murine leukaemic lymphoblasts L5178Y-S *in vitro* and in a slowly growing subline A 651/3 isolated from an X-irradiated L5178Y-S culture and bearing a heritable impairment of proliferative ability.

## MATERIALS AND METHODS

*Material.* Murine leukaemic lymphoblasts L5178Y-S were cultured as suspensions in the Fischer medium supplemented with 8% of calf serum (Alexander & Mikulski, 1961; Szumiel *et al.*, 1971).

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The slowly growing subline A 651/3 was isolated from an irradiated L5178Y-S cell population by cloning, as described previously (Beer *et al.*, 1970).

*Labelled protein and DNA synthesis.* For labelling of protein, the cells were cultured for 10 mean doubling times in growth medium containing 0.17 μCi of L-[1-$^{14}$C]leucine (spec. act. 62 mCi/mmol) per ml of growth medium. Adequate dilutions were performed to keep the cultures within the cell density range allowing exponential growth ($2.3\times10^{4}$ - $8\times10^{5}$/ml). For DNA labelling, the cells were cultured for 3 h in growth medium containing 0.1 μCi of [6-$^{3}$H]thymidine (spec. act. 28 Ci/mmol) per ml of growth medium.

At the end of the labelling period the cells were centrifuged, resuspended in unlabelled growth medium (zero time) and allowed to continue the exponential growth. Samples of the cells were taken at various time intervals for the preparation of histones or assay of DNA radioactivity.

Histone preparations were obtained from isolated nuclei (Pogo *et al.*, 1966) from about $5\times10^{7}$ cells, as described previously (Szumiel *et al.*, 1972).

*Polyacrylamide-gel electrophoresis.* Electrophoresis of histones was performed by the modified method of Senshu (1971) in a vertical slab apparatus as described by Szumiel *et al.* (1974). Ethylene diacrylate was used as a cross-linking factor, giving a gel readily dissolving in concentrated ammonia. The gels were faintly stained with 0.5% Amido Black 10B. This method enables separation of histones into four fractions, as shown in Fig. 1.

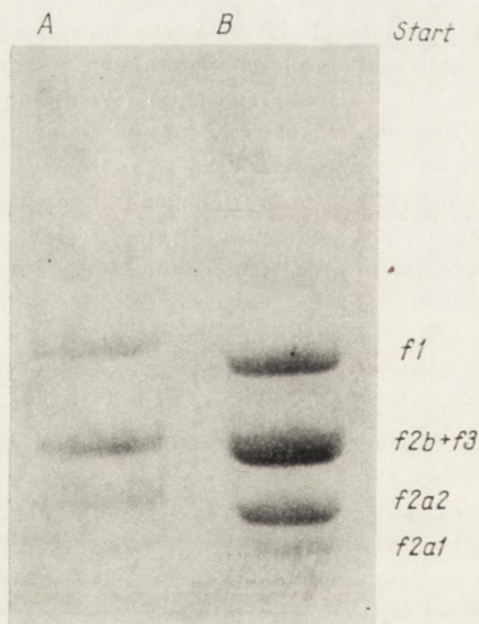


Fig. 1. Polyacrylamide-gel electrophoretic separation of histones from: *A*, murine leukaemic lymphoblasts L5178Y-S; B, calf thymus.

*Analytical methods.* Determinations of DNA were carried out by the method of Schneider (1946) using calf thymus DNA as a standard. Protein in the total histone preparations was determined by the method of Lowry *et al.* (1951) with

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calf thymus histones as a standard. Electrophoretically separated histone fractions were determined by the method of Johns (1967).

*Radioactivity measurements.* Radioactivity of total histone and histone fractions (eluted from gel slices with 0.5 ml of conc. ammonia), was measured as described by Wannemacher (1965), using a scintillation fluid containing dioxane, PPO, POPOP and naphthalene (Hattori *et al.*, 1965). DNA radioactivity was measured by the method of Hattori *et al.* (1965). In all assays Intertechnique (France) SL 30 scintillation counter was used. Sufficient counts were recorded to reduce the counting error to less than 1%. All determinations were carried out in duplicate. Bovine serum proteins and calf thymus DNA, respectively, were added as carriers to adjust protein content to 1 mg and DNA content to 100 μg per sample.

*Reagents.* The following special reagents were used: ethylene diacrylate (Pfaltz & Bauer, Flushing, N.Y., U.S.A.), acrylamide and *N,N'*-bisacrylamide (Fluka AG, Buchs SG, Switzerland); TEMED, PPO and POPOP (Koch-Light, Colnbrook, England); calf thymus DNA and bovine serum albumin (B.D.H., Poole, England); 4-phenylazo-1-naphthylamine (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.). Labelled compounds were supplied by The Radiochemical Centre (Amersham, England). Other reagents were purchased from P.O.Ch. (Gliwice, Poland).

## RESULTS

L5178Y-S cells grown in the radioactive medium were transferred to non-radioactive medium and allowed to continue exponential growth. Under these conditions specific radioactivity of histones decreased due to dilution of the pre-

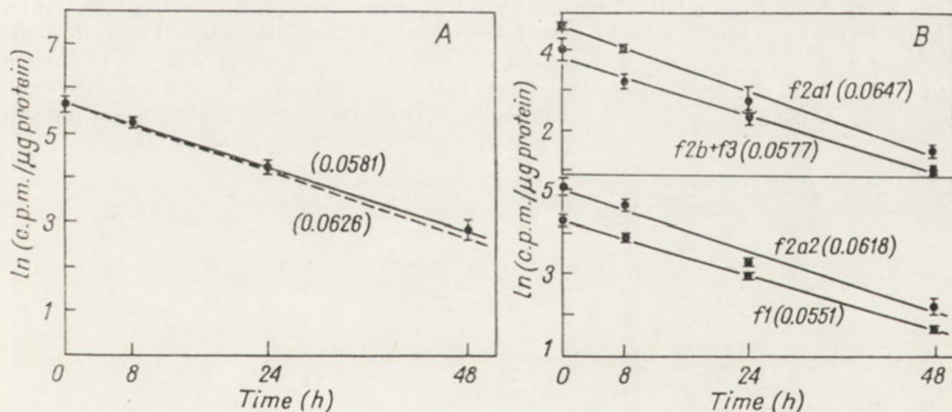


Fig. 2. Decrease of radioactivity of *A*, prelabelled total histone, and *B*, the indicated histone fractions, in exponentially growing L5178Y-S cells (doubling time 11 h). The results are mean values ±S.E.M. from 6 determinations in *A* and 4 in *B*. The experimental (solid) lines were drawn by the least squares method. The broken line illustrates rate of dilution of the prelabelled total histone predicted from the growth rate. Rate constants are indicated in parentheses. In *A*, variance estimated for deviations from linearity is 0.2504. The difference between the slopes of both lines is not significant, at 14 degrees of freedom and the confidence level $P=0.01$. In *B*, rate constants of specific radioactivity dilution obtained for histone fractions are within confidence limits of the rate constant determined for the total histone (0.0581±0.0113 at 11 degrees of freedom and $P=0.05$).

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labelled proteins with the newly synthesized non-radioactive histones. From the measured doubling time of the culture (11.0 h), the specific radioactivity was predicted to decrease at a rate constant of 0.0626. If prelabelled histones were lost from the cells as a result of turnover, the specific radioactivity should decrease at a higher rate. However, no significant difference between the predicted and observed rate of radioactivity dilution has been found (Fig. 2A).

To investigate the turnover of individual histone fractions the histones were separated electrophoretically on polyacrylamide gel. The radioactivity determined in the 4 fractions decreased at a rate which did not significantly differ from that observed for total histone (Fig. 2B).

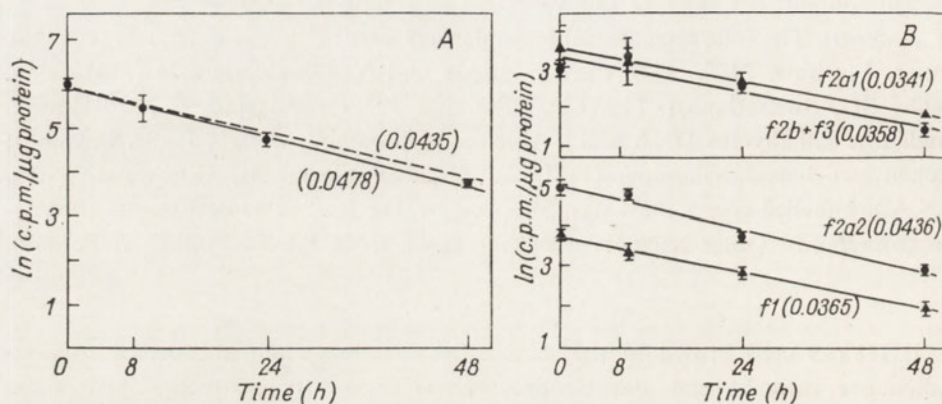


Fig. 3. Decrease of radioactivity of *A*, prelabelled total histone, and *B*, the individual histone fractions, in the slowly growing L5178Y-S subline 651/3, growing exponentially (doubling time 16.4 h). The results are mean values ±S.E.M. from 4 determinations. Explanations as in Fig. 2. In *A*, the difference between rate constants predicted and measured is not significant at 14 degrees of freedom and $P=0.01$. In *B*, rate constants obtained for the individual histone fractions are within the confidence limits of the constant determined for total histone (0.0478±0.0153 at 7 degrees of freedom and $P=0.05$).

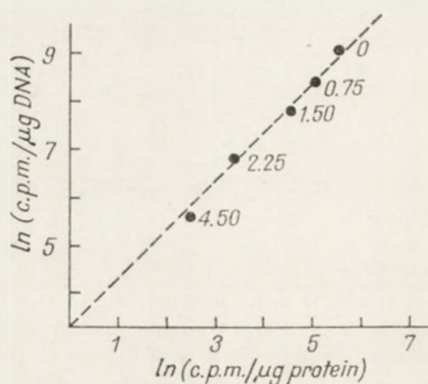


Fig. 4. The rate of change of specific radioactivities of prelabelled DNA and total histone. Measurements were performed at the indicated doubling times of the L5178Y-S cell population. The broken line at the 45° angle represents the theoretical relationship between radioactivity changes of DNA and histone on the assumption of lack of turnover of these compounds.

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In Fig. 3 are presented the results for total histone and the particular histone fractions obtained from the slowly growing subline 651/3, the doubling time for which exceeded that of control by 49% ($T_D$ 16.4 h). The observed rate constant for the decrease in specific radioactivity of total histone was 0.0478, and the rate constant predicted from the growth rate, 0.0435 (Fig. 3A).

To compare the radioactivity dilution rates of total histone and DNA, the decrease in DNA radioactivity was measured at the same time intervals in the cells prelabelled with [$^3$H]thymidine. It was found that the rate of radioactivity decrease was practically the same for both compounds (Fig. 4).

## DISCUSSION

The results obtained for the L5178Y-S cell population indicate that L5178Y-S histones are conserved in the chromatin, at least for 4 cell generations, and that dilution of the label is due entirely to the replication of the chromatin. Equal dilution rates for DNA and histones are in agreement with the view of several authors (Piha *et al.*, 1966; Byvoet, 1966; Hancock, 1969; Murthy *et al.*, 1970; Balhorn *et al.*, 1972) that histones, similarly as DNA, exhibit metabolic stability.

The same conclusion can be drawn from the results obtained for the slowly growing subline A 651/3. Our recent studies indicate that growth retardation in sublines derived from irradiated L5178Y-S cell cultures is connected with a transient arrest in G2 phase of the cell cycle (Beer *et al.*, 1974). It is known that growth of cell populations can be arrested in G2 phase immediately after exposure to X-rays. In the arrest of this kind, called the mitotic delay, Gurley *et al.* (1970, 1971) observed disturbances of histone *f1* and *f3* turnover in a Chinese hamster ovary cell population. However, we have shown that in the case of the slowly growing subline of murine leukaemic lymphoblasts no histone turnover occurs, similarly as in the normally growing L5178Y-S cell population.

Therefore, it seems that histone molecules, similarly as DNA, are conserved in the chromatin, and that X-irradiation resulting in the heritable damages of the proliferative ability does not change the stability of histones.

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## BRAK OBROTU HISTONÓW W NORMALNYCH I DZIEDZICZNIE USZKODZONYCH MYSICH LIMFOBLASTACH BIAŁACZKOWYCH L5178Y-S

### Streszczenie

Stosując wstępne znakowanie [$^{14}$C]leucyną oraz elektroforetyczne frakcjonowanie na żelu poliakrylamidowym stwierdzono brak obrotu całkowitych histonów i ich frakcji *f1*, *f2a1*, *f2a2* i *f2b+f3* zarówno w szybkorosnącej populacji mysich limfoblastów białaczkowych L5178Y-S, jak i wolnorosnącej podlinii wydzielonej z hodowli komórek L5178Y-S napromienionej promieniowaniem X.

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# PREPARATION AND PROPERTIES OF *O'*-METHYL AND *O'*-ETHYL DERIVATIVES OF *1-β-*D-ARABINOFURANOSYLCYTOSINE AND *1-β-*D-ARABINOFURANOSYLURACIL*

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1. Alkylation of 1-β-D-arabinofuranosylcytosine (araC) with dimethylsulphate and diethylsulphate in strongly alkaline medium, followed by chromatography on a basic ion exchange column, was employed to obtain all the possible *O'*-methyl and *O'*-ethyl derivatives of araC in good yields. 2. All the analogous *O'*-alkyl derivatives of 1-β-D-arabinofuranosyluracil (araU) were obtained by deamination of the corresponding araC analogues. Deamination in 1 M-$CH_3COOH$ was shown to be particularly convenient for this purpose; and some preliminary data on the mechanism of the deamination reaction under these conditions, and the kinetics of the reaction, are presented. 3. Identification of the fourteen *O'*-alkyl derivatives of araC was based largely on p.m.r. spectral data, and supported by deamination rates in $CH_3COOH$. Identification of the araU derivatives followed from that for the corresponding araC analogues. 4. Some conclusions are drawn regarding the relative acidities of the three sugar hydroxyls from the sequence of elution of the various *O'*-alkyl derivatives on a basic ion exchange column. The elution sequence, together with u.v. data in alkaline medium for some of the derivatives, point to the existence of hydrogen bonding between the 2'-OH and 5'-OH.

We have previously shown that the relative resistance to alkylation of the ring $N_3$ of 1-substituted cytosines in strongly alkaline medium provides a practical route to the alkylation of the sugar hydroxyls of cytosine nucleosides (Kuśmierek & Shugar, 1971) and, by subsequent deamination, to the corresponding *O'*-alkylated derivatives of uracil nucleosides. A detailed description of this procedure to the synthesis of *O'*-methyl and *O'*-ethyl derivatives of cytidine and uridine has been

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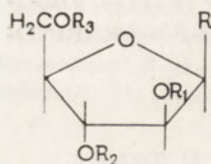
elsewhere presented (Kuśmierek *et al.*, 1973), as well as its extension to nucleotides (Kuśmierek & Shugar, 1973). We have also described in preliminary form (Darżynkiewicz *et al.*, 1972) the application of the foregoing to the methylation of the sugar hydroxyls of 1-β-D-arabinofuranosylcytosine (araC)[1], a nucleoside with interesting antimetabolic properties, which does not contain a *cis*-glycol system on the carbohydrate ring. The present communication deals with considerably extended studies on methylation of nucleosides to obtain all the possible *O'*-methyl derivatives of araC, as well as the analogous *O'*-ethyl derivatives and the corresponding araU analogues (Scheme 1). The mechanism of the alkylation reaction itself has been discussed in detail elsewhere (Kuśmierek & Shugar, 1973).




Scheme 1. Schematical representation of the various *O'*-methyl and *O'*-ethyl derivatives of araC and araU. R=cytosine or uracil. $R_1$, $R_2$ and $R_3$=H, $CH_3$ or $C_2H_5$. For example, if R=cytosine, $R_1=CH_3$, $R_2$=H and $R_3=CH_3$, then the resulting derivative is 2',5'-di-*O*-methyl-araC (2',5'-diOMe-araC).

## RESULTS AND DISCUSSION

Initial attempts to obtain the methyl ether derivatives of araC were based on the use of diazomethane in methanolic solution in the presence of catalytic amounts of $SnCl_2$ (Aritomi & Kawasaki, 1970). This procedure leads to specific methylation of the 2'-OH and 3'-OH of cytidine and adenosine (Robins & Naik, 1971), and has been extended to obtain the 2'- and 3'-*O*-methyl and *O'*-benzyl derivatives of all the "common" nucleosides (Christensen & Broom, 1972). This procedure is very convenient since alkylation proceeds in almost quantitative yield to give the two monoalkylated derivatives, with minimal alkylation of the ring $N_3$ nitrogens (Kuśmierek *et al.*, 1973).

The foregoing procedure, however, gave negative results with araC, as might have been anticipated from the failure of 2'-deoxycytidine to react (Robins & Naik, 1971). The absence of any reaction with araC is obviously due to the fact that the 2'-OH and 3'-OH in this cytidine analogue are *trans* to each other. Direct trials with deoxycytidine, 1-β-D-xylofuranosylcytosine and 1-β-D-lyxofuranosylcytosine demonstrated a reaction only for the latter, which contains a *cis*-glycol system, with formation of approximately equal proportions of the 2'-*O*-methyl and 3'-*O*-methyl derivatives in about 80% yield. The fact that the yield in this case was not quantitative is probably due to the fact that the 2'-OH and 3'-OH groups are both in the "up" form, so that attack by the methylating reagent is subject to steric hindrance by the aglycone.

[1] The following abbreviations are used in this text: araC, 1-β-D-arabinofuranosylcytosine; araU, 1-β-D-arabinofuranosyluracil; 2'-OMe-araC, 2'-*O*-methyl-araC; 3',5'-diOMe-araC, 3',5'-di-*O*-methyl-araC; 2',3',5'-triOEt-araU, 2',3',5'-tri-*O*-ethyl-araU; and similarly for other *O'*-alkyl derivatives of araC and araU (see Scheme 1). DSS, sodium 2,2-dimethyl-2-silapentane-5-sulphonate.

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Table 1

*Paper chromatography, ascending, of O'-methyl and O'-ethyl derivatives of araC and araU*

Whatman paper no. 1 with the following solvent systems (all proportions v/v): (*A*), *n*-butanol - water, 84:16; (*B*), *n*-butanol - acetic acid - water, 5:2:3; (*C*), isopropanol - conc. $NH_4OH$ (d=0.88) - water, 7:1:2; (*D*), ethanol - 0.5 M-ammonium acetate, 5:2.

| Compound | $R_F$ value with solvent | | | |
|---|---|---|---|---|
| | *A* | *B* | *C* | *D* |
| araC | 0.17 | 0.52 | 0.56 | 0.64 |
| 2'-OMe- | 0.37 | 0.63 | 0.68 | 0.76 |
| 2'-OEt- | 0.51 | 0.70 | 0.76 | 0.82 |
| 3'-OMe- | 0.37 | 0.65 | 0.69 | 0.72 |
| 3'-OEt- | 0.52 | 0.77 | 0.75 | 0.79 |
| 5'-OMe- | 0.32 | 0.66 | 0.68 | 0.72 |
| 5'-OEt- | 0.47 | 0.71 | 0.75 | 0.79 |
| 2',3'-DiOMe- | 0.60 | 0.76 | 0.79 | 0.83 |
| 2',3'-DiOEt- | 0.77 | 0.88 | 0.87 | 0.89 |
| 2',5'-DiOMe- | 0.60 | 0.74 | 0.78 | 0.85 |
| 2',5'-DiOEt- | 0.79 | 0.83 | 0.86 | 0.91 |
| 3',5'-DiOMe- | 0.58 | 0.80 | 0.77 | 0.80 |
| 3',5'-DiOEt- | 0.77 | 0.85 | 0.86 | 0.88 |
| 2',3',5'-TriOMe- | 0.75 | 0.85 | 0.85 | 0.88 |
| 2',3',5'-TriOEt- | 0.88 | 0.92 | 0.93 | 0.94 |
| araU | 0.32 | 0.56 | 0.51 | 0.71 |
| 2'-OMe- | 0.54 | 0.70 | 0.60 | 0.80 |
| 2'-OEt- | 0.67 | 0.77 | 0.68 | 0.85 |
| 3'-OMe- | 0.54 | 0.70 | 0.61 | 0.78 |
| 3'-OEt- | 0.68 | 0.80 | 0.72 | 0.82 |
| 5'-OMe- | 0.50 | 0.68 | 0.61 | 0.78 |
| 5'-OEt- | 0.62 | 0.77 | 0.69 | 0.83 |
| 2',3'-DiOMe- | 0.71 | 0.79 | 0.66 | 0.84 |
| 2',3'-DiOEt- | 0.84 | 0.88 | 0.75 | 0.91 |
| 2',5'-DiOMe- | 0.67 | 0.80 | 0.67 | 0.87 |
| 2',5'-DiOEt- | 0.86 | 0.91 | 0.81 | 0.93 |
| 3',5'-DiOMe- | 0.71 | 0.80 | 0.68 | 0.84 |
| 3',5'-DiOEt- | 0.85 | 0.92 | 0.79 | 0.91 |
| 2',3',5'-TriOMe- | 0.83 | 0.88 | 0.75 | 0.89 |
| 2',3',5'-TriOEt- | 0.92 | 0.92 | 0.86 | 0.96 |

The above findings, together with those for methylation under the same conditions of *O*- and *C*-gluco- and galacto-pyranosides (Aritomi & Kawasaki, 1970), point to the high specificity of this catalytic reaction with respect to the mutual orientation of neighbouring hydroxyls. The catalytic activity of $SnCl_2$ is probably due to complex formation with *cis*-hydroxyls. $Sn^{2+}$, as a strong Lewis acid, forms a coordination complex with the free electron pairs on the OH oxygens, leading to an increase in acidity of the latter with a resultant increase in their susceptibility to electrophilic attack by $CH_3^+$ in the reaction with diazomethane.

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The situation is quite different when etherification of the sugar hydroxyls is based on mild alkylation with dialkylsulphate in alkaline medium (Kuśmierek & Shugar, 1971, 1973) as previously demonstrated for methylation of araC (Darżynkiewicz *et al.*, 1972).

*Alkylation of araC.* Methylation of araC involved the portionwise addition of dimethylsulphate to an aqueous alkaline solution, with simultaneous addition of KOH so that the concentration of the latter did not fall below 1 M. The course of the reaction was followed by paper chromatography with solvent system *A* (see Table 1, above), which clearly resolved the three monomethyl derivatives, the three dimethyl derivatives, the trimethyl derivative and unreacted araC (Table 1). The reaction was terminated when the proportions of these derivatives were ~10%, ~25%, ~25% and ~40%, respectively.

Ethylation of araC required somewhat more forcing conditions. Application of a twofold increase in the quantity of alkylating reagent (diethylsulphate) led to formation of about 5% of the tri-*O'*-ethyl-araC, ~20% of the three di-*O'*-ethyl isomers, ~35% of the mono-*O'*-ethyl isomers, and ~40% unreacted araC. These four fractions were also readily resolved by paper chromatography (Table 1). Under

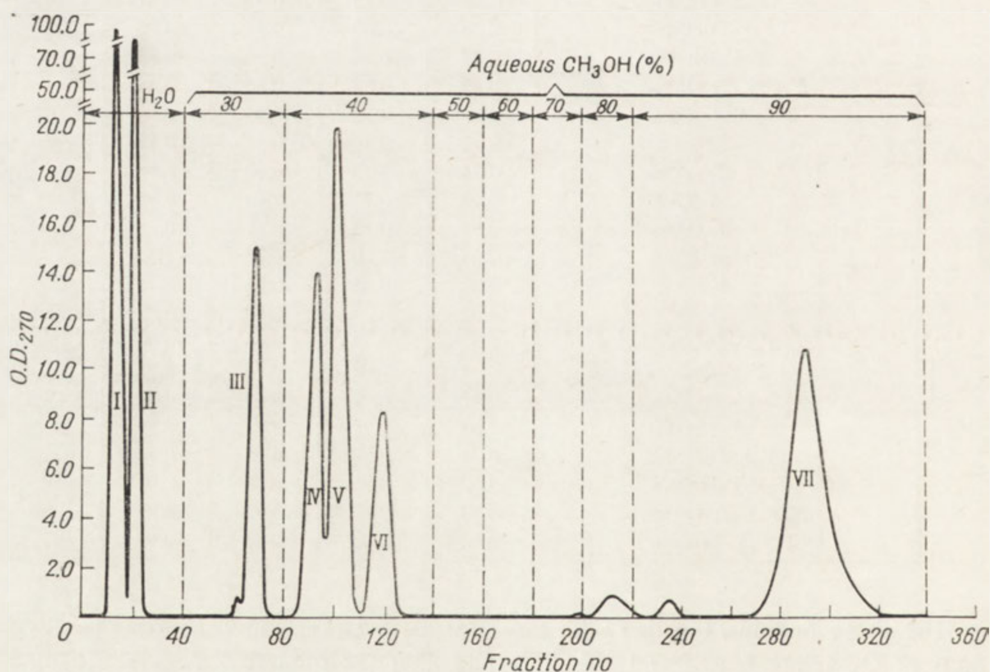


Fig. 1. Elution pattern, on a 60×6.5 cm column of Dowex 1X2 (OH⁻), 200/400 mesh, of products of methylation of araC with dimethylsulphate in alkaline medium as described in Experimental. Fractions of 100 ml collected at 10 min intervals. Elution was with increasing concentrations of aqueous methanol as indicated. 158 000 $OD^{pH 7}_{270}$ units deposited on column: Peak I (2',3',5'-triOMe-araC, 21 000 OD units), II (2',3'-diOMe-araC, 21 000 OD units), III (3',5'-diOMe-araC, 6200 OD units), IV (2',5'-diOMe-araC, 6500 OD units), V (2'-OMe-araC, 11 600 OD units), VI (5'-OMe-araC, 6400 OD units), VII (3'-OMe-araC, 15 000 OD units).

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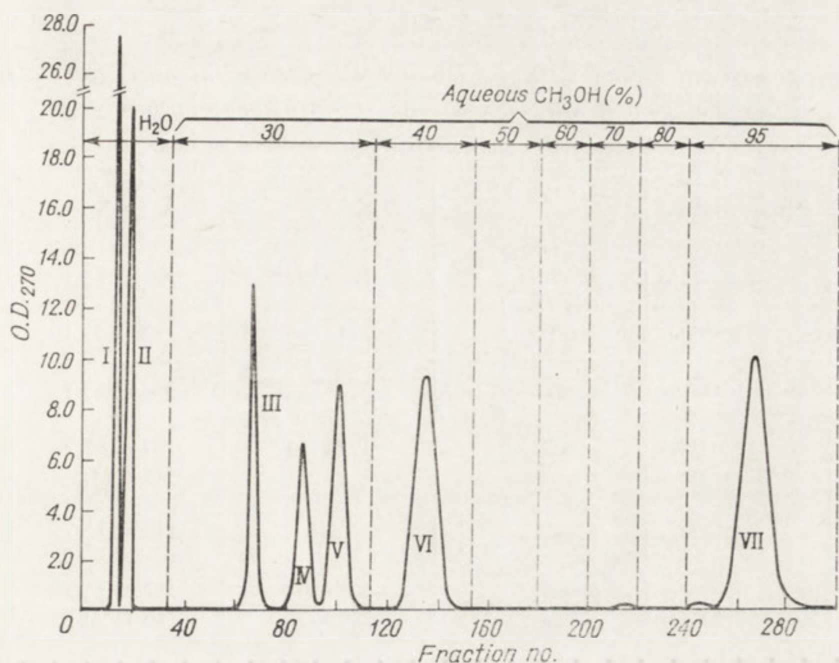


Fig. 2. Elution pattern of products of ethylation of araC with diethylsulphate in alkaline medium on a Dowex (OH⁻) column as described in Fig. 1. 68 000 $OD_{270}^{pH 7}$ units of ethylated araC deposited on column: Peak I (2',3',5'-triOEt-araC, 4280 OD units), II (2',3'-diOEt-araC, 4160 OD units), III (3',5'-diOEt-araC, 5020 OD units), IV (2',5'-diOEt-araC, 3160 OD units), V (2'-OEt-araC, 5380 OD units), VI (5'-OEt-araC, 8500 OD units), VII (3'-OEt-araC, 9480 OD units).

the conditions applied here, virtually no alkylation of the ring $N_3$ or the exocyclic amino group was observed.

Following alkylation, the reaction mixtures were neutralized, freed from salt and subjected to fractionation on a strongly basic Dowex ($OH^-$) column (Dekker, 1965), as described in Experimental. Figures 1 and 2 exhibit the fractionation patterns of the methylated and ethylated reaction mixtures, respectively. In each instance a complete separation of all the alkylated isomers was obtained, facilitating the subsequent isolation and crystallization (in some instances only as the HCl salts) of the fourteen *O'*-methyl and *O'*-ethyl araC derivatives (Tables 2 and 3).

It is pertinent to consider the yields of the individual *O'*-alkyl derivatives of araC with reference to the conformation of the latter, the mechanism of alkylation, and possible steric effects. The alkylation yields were compared with those for cytidine (Kuśmierek *et al.*, 1973) and 1-*β*-D-methylarabinofuranoside (Mied & Lee, 1972) (Table 4).

Alkylation with dialkylsulphate in alkaline medium involves two competing effects in relation to the nucleophilic character of the sugar hydroxyls, *viz.* the relative ease of ionization of a given hydroxyl (degree of acidity) facilitates attack by the alkylating reagent, while the nucleophilic reactivity of the resulting anion increases with its basicity (Koskikallio, 1972; Kuśmierek & Shugar, 1973), i.e.

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Table 2

*Melting points and overall yields of O'-methyl derivatives of araC from a typical methylation with dimethylsulphate in alkaline medium*

All compounds crystallized from anhydrous ethanol.

| AraC derivative | Crystal form | m.p. (°C) | Yield (mg) |
|---|---|---|---|
| 2′-OMe | needles (HCl salt) | 195 - 197 | 390 (7.7%) |
| 3′-OMe- | small prisms | 264 - 266 | 170 (3.4%) |
| 3′-OMe- | needles (HCl salt) | 217 - 221 | 364 (7.2%) |
| 5′-OMe- | plates | 192 - 193 | 190 (3.8%) |
| 2′,3′-DiOMe- | large prisms | 211 - 213 | 545 (11%) |
| 2′,5′-DiOMe- | irregular prisms | 163 - 166 | 110 (2.2%) |
| 3′,5′-DiOMe- | plates | 203 - 205 | 177 (3.5%) |
| 2′,3′,5′-TriOMe- | prisms | 229 - 231 | 146 (3%) |

Table 3

*Melting points and overall yields of O'-ethyl derivatives of araC from a typical ethylation with diethylsulphate in alkaline medium*

The 2′,3′-diOEt- and 2′,3,′5′-triOEt- derivatives were crystallized from ethyl acetate - ethanol, other compounds from anhydrous ethanol.

| AraC derivative | Crystal form | m.p. (°C) | Yield (mg) |
|---|---|---|---|
| 2′-OEt- | parallelopipeds | 194 - 195 | 143 (5.6%) |
| 3′-OEt- | prisms | 242 - 243.5 | 171 (6.2%) |
| 5′-OEt- | long needles | 205 - 205.5 | 245 (9.5%) |
| 2′,3′-DiOEt- | prisms (HCl salt) | 179 - 182 | 126 (4.5%) |
| 2′,5′-DiOEt- | prisms (HCl salt) | 192 - 195 | 67.3 (2.5%) |
| 3′,5′-DiOEt- | prisms (HCl salt) | 183 - 186 | 105 (4%) |
| 2′,3′,5′-TriOEt- | prisms (HCl salt) | 202 - 204 | 130 (4.6%) |

with its decreased acidity. The overall result is an appreciable yield of the 5′-*O*-alkyl derivatives (Table 4), notwithstanding the different acidities of the 2′-OH, 3′-OH and 5′-OH (see below), undoubtedly as a result of mutual compensation of these two effects. The simultaneous existence of both these effects makes it rather difficult to relate the yields of individual derivatives in terms of the reaction mechanism.

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Table 4

*Percentage yield of various O'-alkyl derivatives of cytidine, araC and β-D-methylarabinofuranoside on alkylation in alkaline medium*

For the nucleosides the individual values were obtained from the number of $OD_{270}$ units in the various peaks of the elution pattern on a Dekker column as described in the text. The data for β-D-methylarabinofuranoside are from Mied & Lee (1972), using the methylation procedure of Kuhn & Trishmann (1963). The data for cytidine are from Kuśmierek *et al.* (1973).

| Derivative | O'-Me-araC (%) | | O'-Et-araC (%) | | O'-Me-cytidine (%) | | O-Me-β-D-arabinofuranoside (%) | |
|---|---|---|---|---|---|---|---|---|
| 2'- | 7.3 | 20.7 | 8.0 | 34.5 | 32 | 49 | 4.04 | 5.88 |
| 3'- | 9.4 | | 14.0 | | 8 | | 0.88 | |
| 5'- | 4.0 | | 12.5 | | 9 | | 0.86 | |
| 2',3'- | 13.1 | 20.9 | 6.2 | 18.3 | — | 21 | 22.72 | 29.18 |
| 2',5'- | 3.9 | | 4.7 | | — | | 3.22 | |
| 3',5'- | 3.9 | | 7.4 | | — | | 3.24 | |
| 2',3',5'- | 13.1 | | 6.3 | | 4.0 | | 12.61 | |
| Total (%) | 54.7 | | 59.1 | | 74 | | 47.67 | |

By contrast, there is a clear-cut dependence of the yields of the various *O'*-alkyl derivatives on the nucleoside configuration. The *anti* conformation of the cytosine ring with respect to the pentose ring (Remin & Shugar, 1973) leads to considerable steric hindrance for the "up" 2'-OH, with a consequent lower yield of 2'-*O*-alkyl derivatives than in the case of cytidine and 1-β-D-methylarabinofuranoside (see Table 4). Furthermore the larger bulk of the ethyl group leads, as might have been anticipated, to lower yields of the 2'-*O*-ethyl analogues of araC as compared to the 2'-*O*-methyl analogues (Table 4). In addition ethylation of the 2'-OH (or 5'-OH) hinders to some extent ethylation of the 5'-OH (or 2'-OH). Comparison of the relative yields of the various *O'*-methyl and *O'*-ethyl derivatives of araC, under conditions where the overall yields of products are similar (55 - 60%) shows that the ratio of mono:di:tri *O'*-alkyls is 1.6:1.6:1 for the methyl derivatives and 6:3.3:1 for the corresponding ethyl derivatives.

*Identification of O'-methyl araC derivatives.* Identification of the three mono-*O'*-methyl derivatives of araC, previously described (Darżynkiewicz *et al.*, 1972), was based on chemical evidence, as well as on comparisons with the 5'-*O*-methyl derivative synthesized by an unambiguous route (Giziewicz *et al.*, 1972). These identifications have now been further confirmed by the direct synthesis of 2'-*O*-methyl-araC (Giziewicz & Shugar, 1973), and are also consistent with the p.m.r. spectra of the three monomethylated derivatives.

The chemical shifts of the methyl protons of the three monomethylated derivatives made it possible, in turn, to unambiguously identify the remaining four multiply methylated derivatives. The chemical shifts of the methyl protons for all the *O'*-methyl derivatives of araC are listed in Table 5. It will be noted from the Table that the chemical shifts of the three methyl groups in 2',3',5'-tri-*O*-methyl-araC virtually coincide (to within 0.005 ppm) with those for the three monomethyl

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derivatives. A similar situation prevails for the three di-*O*′-methyl analogues. In all instances the differences between the chemical shifts of the individual methyls in the multiply methylated derivatives differ from those for the corresponding methyls in the monomethylated compounds by only a few thousandths ppm, whereas the difference in chemical shifts between the methyl groups in 2′-*O*-methyl-araC and 3′-*O*-methyl-araC is of the order of several hundredths ppm, hence an order of magnitude greater.

Table 5

*Chemical shifts (in ppm vs internal DSS) of the* H-1′, *methyl and methylene protons of the various O′-methyl and O′-ethyl derivatives of araC in* $D_2O$ *at* pD~*3*

For the 2′- and 3′-ethyl groups, the signals corresponding to the methylene protons (denoted as $H_A$ and $H_B$) have been assigned on the basis of a conformational analysis of the ethyl substituents, details of which will be published elsewhere. For the 5′-*O*-ethyl substituent the A and B methylene protons are indistinguishable.

| *O*′-Alkyl derivatives | H-1′ | 2′-$CH_3$ | 3′-$CH_3$ | 5′-$CH_3$ | 2′-$CH_2$ | | 3′-$CH_2$ | | 5′-$CH_2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | $H_A$ | $H_B$ | $H_A$ | $H_B$ | $H_A \equiv H_B$ |
| araC | 6.199 | — | — | — | | | | | |
| 2′-OMe- | 6.297 | 3.371 | — | — | | | | | |
| 3′-OMe- | 6.113 | — | 3.498 | — | | | | | |
| 5′-OMe- | 6.193 | — | — | 3.455 | | | | | |
| 2′,3′-DiOMe- | 6.192 | 3.371 | 3.513 | — | | | | | |
| 2′,5′-DiOMe- | 6.300 | 3.382 | — | 3.447 | | | | | |
| 3′,5′-DiOMe- | 6.114 | — | 3.502 | 3.466 | | | | | |
| 2′,3′,5′-TriOMe- | 6.198 | 3.366 | 3.505 | 3.449 | | | | | |
| 2′-OEt- | 6.303 | 1.082 | — | — | 3.684 | 3.508 | — | — | — |
| 3′-OEt- | 6.134 | — | 1.225 | — | — | — | 3.773 | 3.707 | — |
| 5′-OEt- | 6.197 | — | — | 1.223 | — | — | — | — | 3.674 |
| 2′,3′-DiOEt- | 6.233 | 1.076 | 1.226 | — | 3.687 | 3.453 | 3.763 | 3.716 | — |
| 2′,5′-DiOEt- | 6.296 | 1.079 | — | 1.215 | 3.662 | 3.519 | — | — | 3.661 |
| 3′,5′-DiOEt- | 6.127 | — | 1.221 | 1.221 | — | — | 3.771 | 3.697 | 3.677 |
| 2′,3′,5′-TriOEt- | 6.232 | 1.081 | 1.224 | 1.224 | 3.668 | 3.464 | 3.772 | 3.702 | 3.661 |

The foregoing comparison is further supported by a detailed analysis of the chemical shifts of the remaining pentose protons (Remin & Shugar, 1973), each of which is subject to the magnetic anisotropy of the *O*′-$CH_3$ bonds ("neighbour anisotropy effect"). A characteristic feature of the modifications in chemical shift of a given proton was the observation that these were independent of whether an additional methyl group was present or not, i.e. the effects on a given proton were fully additive. The most pronounced shielding effect on a given proton was, of course, observed in the presence of a neighbouring methyl group, e.g. a 2′-*O*-methyl substituent led to pronounced shielding of the *geminal* H-2′ by about 0.30 ppm. For purposes of illustration Table 5 exhibits the chemical shifts of the H-1′ protons for all the methylated araC derivatives.

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*Identification of O′-ethyl araC derivatives.* Identification of the three mono-O′-ethyl derivatives from the p.m.r. spectra was relatively straightforward, bearing in mind that ethylation of a given position, e.g. the 2′-OH, led to pronounced shielding of the nearest neighbouring proton, in this case the *geminal* H-2′. Furthermore the influence of a given ethyl substituent on the chemical shift of H-1′ was similar to that of a corresponding methyl group, discussed above (see Table 5). The absence of any change in chemical shift of H-1′ following ethylation of the 5′-OH, which is furthest removed from H-1′, is consistent with identification of 5′-*O*-ethyl-araC as such.

As regards the multiply ethylated derivatives, these were readily distinguished from each other by a comparison of the chemical shifts of the corresponding methyl and methylene protons, the results of which are presented in Table 5. For the compounds ethylated on the 2′-OH and 3′-OH, the methyl and methylene protons exhibit varying chemical shifts. By contrast, the methylene protons of the 5′-*O*-ethyl are magnetically equivalent. The chemical shifts of the methylene protons exhibit characteristic values, depending on the position of ethylation, and are modified to only a minor extent on introduction of a second ethyl substituent. Furthermore the chemical shift values for H-1′ exhibit additivity when a second ethyl substituent is introduced. A more extensive analysis of the spectra of these derivatives is under way, with a view to establishing the conformations of the individual ethyl substituents about the C-*O*′-bonds (Remin, Darżynkiewicz & Shugar, unpublished).

Further evidence for the unequivocal assignments of the various *O*′-ethyl derivatives of araC is forthcoming from a study of the kinetics of deamination of the individual compounds (cf. Darżynkiewicz *et al.*, 1972), described below.

*Acidity of sugar hydroxyls and sequence of elution from Dowex OH⁻.* Fractionation on a strongly basic Dowex $OH^-$ column has been profited from to draw some conclusions as to the relative acidities of the sugar hydroxyls of nucleosides, based on the sequence of elution with aqueous methanol (Dekker, 1965). Methylation of a given hydroxyl leads to a decrease in acidity with a corresponding decrease in binding ability of the nucleoside to the basic resin. The sequence of elution of *O*′-methyl derivatives of adenosine (Gin & Dekker, 1968) and cytidine (Martin *et al.*, 1968) from a Dowex $OH^-$ column led to the conclusion that the relative acidities of the ribose hydroxyls were 5′-OH < 3′-OH < 2′-OH.

The virtually ideal fractionations on Dowex $OH^-$ of all the *O*′-methyl and *O*′-ethyl derivatives of araC (Figs. 1 and 2) permit of certain conclusions regarding the acidities of the hydroxyls of the arabinose ring, apparently free from the complications involved in the case of ribonucleosides, where the 2′-OH and 3′-OH form a *cis*-glycol system which may be hydrogen-bonded (see, however, below). The first two peaks, corresponding to tri-*O*′-alkyl-araC and 2′,3′-di-*O*-alkyl-araC, are rapidly eluted with water. The ready elution of the tri-*O*′-alkyl derivative is consistent with the absence of any free hydroxyls and concomitant inability to bind to the resin. The appearance immediately behind this compound of 2′,3′-di-*O*-

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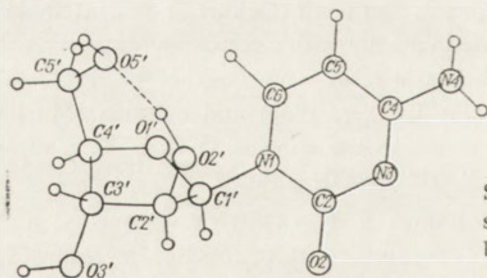
alkyl-araC testifies to the very low acidity of the 5′-OH as in the case of ribonucleosides (Gin & Dekker, 1968).

The subsequent sequence of elution, with 30 - 40% aqueous methanol, is 3′,5′-di-*O*-alkyl, 2′,5′-di-*O*-alkyl, 2′-*O*-alkyl and 5′-*O*-alkyl. In contrast to the foregoing, 3′-*O*-alkyl-araC is so strongly bound to the resin that it is eluted only on raising the methanol concentration to over 90%.

If we attempt to estimate the relative acidities of the hydroxyls of the di-*O*′-alkyl-araC derivatives from the elution sequence, the results from Figs. 1 and 2 suggest that these acidities increase in the order 5′-OH, 2′-OH and 3′-OH for the individual isolated hydroxyl groups. On the other hand the sequence of elution of the mono-*O*′-alkyl derivatives, each of which possesses two free hydroxyls, points to the sequence (increasing acidity) 3′-OH, 5′-OH, 2′-OH. It should be emphasized that blocking of the relatively weakly acid 5′-OH apparently leads to a marked decrease in acidity of the system, notwithstanding the presence of two relatively acid groups, 2′-OH and 3′-OH. By contrast, the derivative with a blocked 3′-OH, and therefore with a free 2′-OH and 5′-OH, exhibits much higher acidity. It follows that any estimate of the relative acidity of a given hydroxyl in the arabinose ring cannot be assessed on the basis of additivity, but only with reference to a given derivative. A more valid conclusion is that the appreciable acidity of araC itself, and its 3′-*O*-alkyl derivatives, is due largely to the presence of the free 2′-OH and 5′-OH, which may hydrogen bond when the conformation of the exocyclic 5′-$CH_2OH$ is *gauche-gauche* (Scheme 2). This would be somewhat analogous to the hydrogen bonding proposed by Izatt *et al.* (1965) between the *cis*-hydroxyls of ribose, and derives support from the demonstration that in the crystalline state such a hydrogen bond between the 5′-OH and 2′-OH in araC (with the 2′-OH as the donor) is geometrically feasible (Chwang & Sundaralingam, 1973). The results showed that the distance O(2′)-H···O(5′) in the crystal is 2.64 Å, the 5′-$CH_2OH$ being exclusively in the form *gauche-gauche*.




Scheme 2. Molecular conformation in the solid state of araC, showing intramolecular hydrogen bonding between the 2′-OH and the 5′-O (from Chwang & Sundaralingam, 1973).

*U.v. absorption spectra.* Spectral data for all the crystalline *O*′-alkyl derivatives of araC and araU are presented in Table 6 (see also Fig. 3). In neutral and acid medium these are very similar to those for the parent araC and araU. An examination of the u.v. absorption spectra of *O*′-alkyl derivatives of araC in alkaline medium (0.01 - 1 M-NaOH), where spectral changes result from dissociation of the sugar

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Table 6

*U. v. spectra for various O'-methyl and O'-ethyl analogues of araC and araU*

| Derivative of araC or araU | pH | $\lambda_{max}$ (nm) | $\varepsilon_{max}$ ($\times 10^{-3}$) | $\lambda_{min}$ (nm) | $\varepsilon_{min}$ ($\times 10^{-3}$) |
|---|---|---|---|---|---|
| araC | 2 | 280 | 13.5 | 241 | 1.5 |
| | 12 | 272 | 9.7 | 250 | 5.9 |
| 2'-OMe- | 2 | 280 | 13.1 | 241 | 1.6 |
| | 12 | 271 | 9.5 | 250 | 6.5 |
| 2'-OEt- | 2 | 280 | 13.2 | 241 | 1.7 |
| | 12 | 271 | 9.2 | 250 | 6.1 |
| 3'-OMe- | 2 | 280 | 13.1 | 240 | 1.3 |
| | 12 | 272 | 9.3 | 250 | 5.6 |
| 3'-OEt- | 2 | 280 | 13.0 | 240 | 1.3 |
| | 12 | 272 | 9.6 | 250 | 6.0 |
| 5'-OMe- | 2 | 280 | 13.5 | 241 | 1.9 |
| | 12 | 271 | 9.4 | 250 | 6.0 |
| 5'-OEt- | 2 | 280 | 13.1 | 240 | 1.3 |
| | 12 | 271 | 9.7 | 250 | 6.2 |
| 2',3'-DiOMe- | 2 | 280 | 13.2 | 240 | 1.6 |
| | 12 | 271 | 9.0 | 250 | 5.9 |
| 2',3'-DiOEt- | 2 | 280 | 13.7 | 241 | 1.9 |
| | 12 | 272 | 9.4 | 250 | 6.2 |
| 2',5'-DiOMe- | 2 | 280 | 13.1 | 240 | 1.8 |
| | 12 | 271 | 9.2 | 250 | 6.3 |
| 2',5'-DiOEt- | 2 | 280 | 14.0 | 241 | 1.8 |
| | 12 | 272 | 9.9 | 250 | 6.5 |
| 3',5'-DiOMe- | 2 | 280 | 13.6 | 241 | 1.5 |
| | 12 | 271 | 9.6 | 250 | 6.0 |
| 3',5'-DiOEt- | 2 | 280 | 13.3 | 241 | 1.6 |
| | 12 | 272 | 8.8 | 250 | 5.7 |
| 2',3',5'-TriOMe- | 2 | 280 | 14.4 | 241 | 1.8 |
| | 12 | 271 | 10.1 | 250 | 6.3 |
| 2',3',5'-TriOEt- | 2 | 280 | 14.2 | 240 | 1.6 |
| | 12 | 272 | 9.8 | 250 | 6.4 |
| araU | 2 | 262 | 9.9 | 230 | 2.0 |
| | 12 | 262 | 7.4 | 241 | 4.5 |
| 2'-OMe- | 2 | 262 | 10.7 | 230 | 2.2 |
| | 12 | 262 | 8.0 | 242 | 5.4 |
| 2'-OEt- | 2 | 262 | 9.9 | 231 | 2.5 |
| | 12 | 262 | 7.4 | 241 | 5.1 |
| 3'-OMe- | 2 | 262 | 10.6 | 230 | 2.7 |
| | 12 | 262 | 8.0 | 241 | 5.1 |
| 5'-OEt- | 2 | 262 | 10.1 | 230 | 2.0 |
| | 12 | 262 | 7.7 | 241 | 4.8 |
| 2',3',5'-TriOMe- | 2 | 262 | 10.1 | 230 | 1.9 |
| | 12 | 262 | 7.7 | 241 | 5.3 |

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hydroxyls (Fox & Shugar, 1952), leads to conclusions in agreement with those derived from the elution sequence on the basic resin column. The spectra of both 3′-*O*-methyl-araC and 3′-*O*-ethyl-araC are most sensitive to pH and exhibit appreciable modifications at pH 12; the spectra at pH 13 are almost identical with those in 1 M-NaOH, clearly demonstrating that the free hydroxyl(s) are almost fully ionized at pH 13 (Fig. 3). Indeed, it may be assumed from the foregoing that the

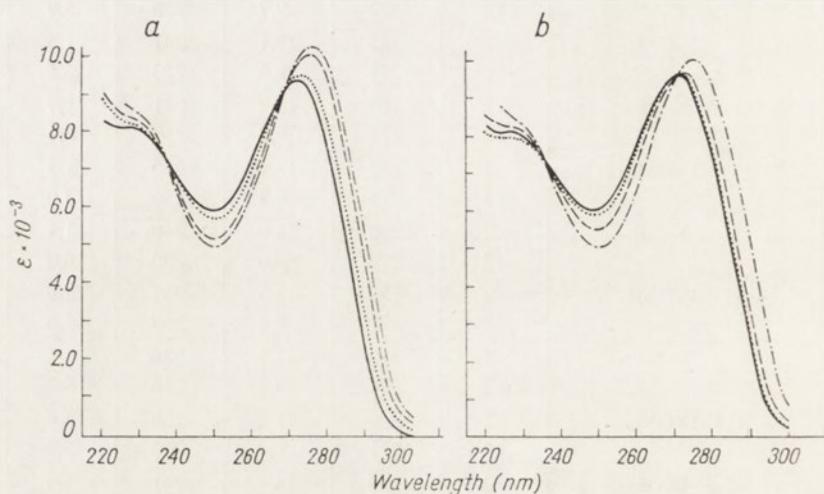


Fig. 3. U.v. absorption spectra of (*a*) 3′-OMe-araC and (*b*) 3′,5′-diOMe-araC, at pH 7 (——), in 0.01 M-NaOH (···), in 0.1 M-NaOH (- - -) and in 1 M-NaOH (-·-·-).

p*K* for dissociation of the system 2′-OH - - - 5′-OH is in the neighbourhood of 12.5, hence similar to that for the system 2′-OH - - - 3′-OH in the corresponding riboside (Izatt *et al.*, 1965; Christensen *et al.*, 1967). Upon additional alkylation of the 5′-OH, the resulting 3′,5′-di-*O*-alkyl derivative exhibits a modification in the u.v. spectrum only at pH 13, so that the p*K* of the isolated 2′-hydroxyl is probably in the neighbourhood of 13.5 (Fig. 3). The spectral changes of 2′,5′-di-*O*-alkyl-araC and 2′-*O*-alkyl-araC are similar and suggest a p*K* for the isolated 3′-OH of about 13, whereas that for the 5′-OH in 2′,3′-di-*O*-alkyl derivatives appears to be above 14. Further studies on pentose hydroxyl ionization by means of u.v. and p.m.r. spectroscopy are now under way.

*Deamination of O′-alkyl derivatives of araC.* Deamination of cytosine nucleosides in aqueous buffered medium in the pH range 2 - 7 has been investigated by Shapiro & Klein (1966, 1967), Notari (1967), Notari *et al.* (1970, 1972) and Garrett & Tsau (1972). For cytidine and araC in acetate buffer, the optimal pH for deamination is 3.6. In the case of cytidine the reaction was postulated to proceed *via* the intermediate addition of the buffer anion to $C_6$ with resultant saturation of the 5,6 bond. For araC which, under analogous conditions, is deaminated at a rate more than 50-fold greater than that for cytidine, the mechanism proposed is intramolecular catalysis by the "up" 2′-OH *via* formation of a cyclic intermediate with $C_6$ (Notari *et al.*, 1972).

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Table 7

*Purification and/or crystallization of O'-alkyl derivatives of araU*

Each of the araU derivatives was obtained by deamination of either the corresponding crystalline araC analogue, or the material remaining in the mother liquors following crystallization of the araC analogue (indicated in 1st column). In those instances where crystallization could not be induced, each product was purified by t.l.c. See text for further details.

| AraU derivative | Amount of araC analogue deaminated | Solvent for crystallization of araU analogue | Melting point and crystal form | Yield (mg) |
|---|---|---|---|---|
| 2'-OMe- | 200 mg from mother liquors | ethyl acetate-ethanol | 153 - 156°C, amorphous | 76 (38%) |
| 3'-OMe- | 150 mg from mother liquors | anhydrous ethanol (-20°C) | 183 - 185°C, irregular prisms | 79 (53%) |
| 5'-OMe- | 100 mg from mother liquors | t.l.c. | Colourless oil | 65 (65%) |
| 2',3'-DiOMe- | 120 mg | t.l.c. | Colourless oil | 64 (53%) |
| 2',5'-DiOMe- | 25 mg | t.l.c. | Colourless oil | 9 (36%) |
| 3',5'-DiOMe- | 60 mg from mother liquors | t.l.c. | Colourless oil | 36 (60%) |
| 2',3',5'-TriOMe- | 250 mg from mother liquors | ethyl acetate-ethanol | 106 - 108°C, amorphous | 67 (27%) |
| 2'-OEt- | 30 mg from mother liquors | ethyl acetate-methanol | 191 - 192°C amorphous | 15 (50%) |
| 3'-OEt- | 28 mg from mother liquors | t.l.c. | Colourless oil | 19 (68%) |
| 5'-OEt- | 50 mg | anhydrous ethanol (-20°C) | 153 - 156°C irregular prisms | 13 (26%) |
| 2',3'-DiOEt- | 30 mg | t.l.c. | Colourless oil | 17 (57%) |
| 2',5'-DiOEt- | 15 mg from mother liquors | t.l.c. | Colourless oil | 8 (53%) |
| 3',5'-DiOEt- | 45 mg from mother liquors | t.l.c. | Colourless oil | 34 (76%) |
| 2',3',5'-TriOEt- | 50 mg from mother liquors | t.l.c. | Colourless oil | 27 (54%) |

We have carried out a detailed study of the kinetics of deamination of all the *O'*-methyl derivatives of araC in acetate buffer, the results of which will be presented elsewhere (Darżynkiewicz, Dudycz & Shugar, unpublished). In full agreement with the mechanism proposed for araC (Notari *et al.*, 1970, 1972), all the derivatives with a free 2'-OH were deaminated at a rate comparable to that for araC itself; whereas those with a 2'-*O*-methyl exhibited rate constants similar to that for cytidine. This not only provides supporting evidence for the two proposed mechanisms of deamin-

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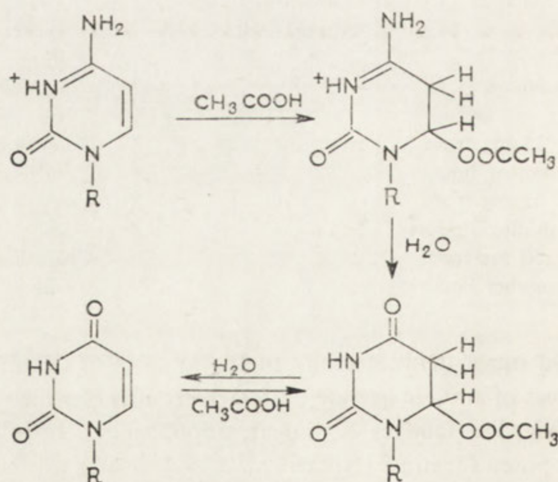
ation, but also a supplementary method for identification of the various *O'*-methyl araC analogues

The foregoing findings suggested that acetic acid might prove a suitable reagent for the preparative deamination of *O'*-alkyl araC derivatives with a free 2'-OH. Actual trials showed that several hours heating at 100°C in 1 M-$CH_3COOH$ sufficed for this purpose. This is by far the most convenient procedure for preparation of araU from araC, and we have previously employed it for preparation of 5'-OMe-araU from 5'-OMe-araC (Giziewicz *et al.*, 1972).

Prolongation of the reaction time to about 5 - 7 days led to 80 - 90% deamination of those *O'*-alkyl araC derivatives with a 2'-*O*-alkyl group, only trace hydrolysis of the glycosidic bonds being observed under these conditions. Consequently deamination in 1M -acetic acid was employed for the preparation of all the fourteen *O'*-alkyl araU derivatives described below. Unreacted *O'*-alkyl-araC was removed by adsorption on Dowex $H^+$. Crystallization of the araU derivatives proved somewhat difficult because of their high solubility and the smaller quantities of material available. Only five of these were isolated in crystalline form; the remainder were obtained as colourless oils following supplementary chromatography on silica gel (Table 7).

The mechanism of deamination in acetic acid for araC derivatives with a blocked 2'-OH is probably similar to that with bisulfite (Shapiro *et al.*, 1970; Hayatsu *et al.*, 1970), involving the intermediate addition of the acetate anion to the cytosine $C_6$ as shown in Scheme 3. Acetate deamination is undoubtedly more convenient, particularly for small-scale deaminations, since subsequent removal of acetic acid is much simpler than of sodium bisulfite. This procedure has also been applied successfully to preparative deamination of 1-*β*-D-lyxofuranosylcytosine.




Scheme 3. Proposed scheme for deamination of 1-substituted cytosines in acetic acid medium. R denotes a sugar moiety. In the event that R is arabinose, this scheme is applicable only if the 2'-OH is blocked.

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## EXPERIMENTAL

The araC used in this study was prepared as described by Kanai *et al.* (1970).

Details of paper chromatography are presented in Table 1. Thin-layer chromatography made use of $7.5 \times 2.5$ cm plates of $HF_{254}$ silica gel. Preparative chromatography on $PF_{254}$ silica gel was carried out with $20 \times 16$ cm plates covered with 16 g gel.

Melting points (uncorrected) were measured on a Boethius hot stage. U.v. spectra were recorded on a Perkin-Elmer Model 450 or a Unicam SP-8000, using 10-mm path length cuvettes. For more accurate measurements a Zeiss (Jena, G.D.R.) VSU-2P manual instrument was employed. P.m.r. spectra were obtained with the aid of a Varian XL-100 instrument (see Table 5 for details).

*O'-Methylation of araC*. To 5.0 g (20.6 mmol) of araC dissolved in 500 ml 1 M-KOH, and vigorously stirred with a teflon-coated magnetic flea, was added 40 ml (400 mmol) of dimethylsulphate in 1-ml portions over a period of 10 h. Simultaneously, in order to maintain the alkalinity of the reaction mixture, 50 ml 10 M-KOH was added in 10-ml portions over the same period. The reaction was terminated by neutralization of the solution with conc. $H_2SO_4$ at the point when thin-layer chromagraphy on $HF_{254}$ silica gel with chloroform - methanol (8:2, v/v) demonstrated the presence of four spots with the following $R_F$ values (and approximate relative intensities under a dark u.v. lamp): 0.15 (40%), unreacted araC; 0.30 (25%), mono-*O'*-methyl-araC; 0.50 (25%), di-*O'*-methyl-araC; 0.65 (10%), tri-*O'*-methyl-araC. The solution was brought almost to dryness under reduced pressure and the residue extracted portionwise with 300 ml methanol, which was filtered to remove inorganic salts. The methanolic solution (171 600 $OD_{270}^{pH7}$ units) was then brought to dryness under reduced pressure. The resultant oily residue was taken up in 700 ml water and deposited on a $35 \times 2.5$ cm column of 200/400 Dowex 50WX2 ($H^+$). The column was washed with water until the effluent gave a neutral reaction, and elution then carried out with 1 M-KOH. The nucleosides were found in the first 150 ml effluent, 160 000 $OD_{270}^{pH7}$ units. To this was added 350 ml water, and the resultant solution passed through a $35 \times 3.5$ cm column of 20/50 Dowex 50 WX2 ($NH_4^+$) to the point where the effluent exhibited no u.v. absorption. The eluate was brought to dryness, with particular attention to removal of ammonia, and the residue taken up in 25 ml water (158 000 $OD_{270}^{pH7}$ units). This was deposited on a $60 \times 6$ cm column of 200/400 Dowex 1X2 ($OH^-$) which had been previously washed with water. Elution, initially with water, and subsequently with increasing concentrations of methanol-water led to the resolution of all the seven possible *O'*-methyl derivatives of araC (cf. Fig. 1). The combined eluates for the individual peaks were brought to dryness and, following establishment of their homogeneity by chromatography (Table 1), were subjected to crystallization from anhydrous ethanol by slow evaporation in a dessicator over $P_2O_5$. In some instances where crystallization did not proceed readily, the HCl salts were obtained in crystalline form as follows: the ethanolic solution was brought to about pH 2 with 2 M-HCl, taken to dryness and excess HCl removed by several evaporations

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with ethanol. The resulting HCl salts were then crystallized from anhydrous ethanol. The final yields of the individual products are given in Table 2.

For the three mono-*O'*-methylated derivatives, elementary analyses supported the assigned structures. The C, H, N values for the 3'-OMe- and 5'-OMe- analogues were respectively: C — 46.16%, 46.30%; H — 5.65%, 5.85%; N — 16.50%, 16.45%; theoretical values for these are: C — 46.69%; H — 5.80%; N — 16.34%. For the HCl salt of 2'-OMe-araC, experimental values were: C — 40.24%, H — 5.60%, N — 14.38%; theory gives C — 40.89%, H — 5.49%, N — 14.38%.

*O'-Ethyl derivatives of araC.* To a continuously stirred solution of 2.5 g (8.9 mmol) of the HCl salt of araC in 500 ml 1 M-KOH was added 40 ml (400 mmol) of diethyl sulphate in stepwise portions of 1 ml over a period of about 12 h. During this same period a total of 50 ml 10 M-KOH was added portionwise to maintain alkaline conditions, as in the methylation procedure in the previous section. The reaction was terminated by neutralization of the solution with conc. $H_2SO_4$ when chromatography on $HF_{254}$ silica gel with chloroform - methanol (8:2, v/v) exhibited four spots with approximate relative intensities under a dark u.v. lamp of 40% unreacted material, 35% mono-*O'*-ethyl-araC, 20% di-*O'*-ethyl-araC and 5% of the tri-*O'*-ethyl derivative (see Table 1 for $R_F$ values). The reaction mixture was then brought to dryness, the residue exhaustively extracted with 300 ml methanol and the extracts freed from salt by filtration. The methanolic solution (78 000 $OD_{270}^{pH7}$ units) was brought to dryness, the resulting oily residue taken up in 500 ml water, and the solution layered on a 35×2.5 cm column of 200/400 Dowex 50 WX2 ($H^+$) which was then washed with water until the effluent exhibited a neutral reaction. Subsequent elution with 150 ml 1 M-KOH gave 67 500 $OD_{270}^{pH7}$ units of nucleoside. The eluate was brought to 500 ml with water and passed through a 35×3.5 cm column of 20/50 Dowex 50 WX8 ($NH_4^+$), followed by elution with water until the effluent was free of u.v. absorbing material. The total eluate (67 000 $OD_{270}^{pH7}$ units) was taken to dryness, the residue dissolved in 20 ml water and the resulting solution deposited on a 60×6 cm column of 200/400 Dowex 1X2 ($OH^-$). The column was then eluted with water, followed by increasing concentrations of aqueous methanol, as shown in Fig. 2. The combined eluates corresponding to the individual peaks in Fig. 2 were brought to dryness and crystallized from anhydrous ethanol or ethyl acetate - ethanol as described above for the methylated derivatives. For several of the derivatives, conversion to the HCl salts was necessary for crystallization. The final yields and properties of the various products are presented in Table 3.

*O'-Alkyl derivatives of araU.* All of the foregoing *O'*-methyl and *O'*-ethyl derivatives of araC were subjected to deamination in 1 M-$CH_3COOH$ (about 20 mg compound/ml acid in sealed ampoules) at 97°C in an ultrathermostat. For those araC derivatives with a free 2'-OH the reaction time was 6 - 8 hours. For most of the others the reaction time was 7 days; and for the 2',5'-di-*O*-alkyl and 2',3',5'-tri-*O*-alkyl derivatives about 10 days was required.

Following completion, or near completion of deamination, the solutions were brought to dryness, then taken up in water and again brought to dryness. The latter operation was repeated several times. The final residue was kept under vacuum

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over KOH pellets to remove traces of $CH_3COOH$. Each of the $O'$-alkyl derivatives was passed through a small Dowex ($H^+$) 50W column to remove unreacted $O'$-alkyl-araC and brought to dryness. The residues were then subjected to crystallization. Those derivatives which could not be crystallized were further purified by chromatography on $16\times20$ cm plates of $PF_{254}$ silica gel with the solvent system methanol - chloroform (2:8, v/v). The products were located under a u.v. lamp, the gel scraped off and extracted with methanol, and the methanol removed to give colourless oils. Details for the various derivatives are presented in Table 7.

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We should like to express our thanks to A. Dworak for running the p.m.r. spectra, to M. Remin for assistance in interpretation, and to L. Dudycz for running some of the u.v. spectra. We are also indebted to Dr. J. J. Fox for a gift of 1-β-D-lyxofuranosylcytosine and 1-β-D-xylofuranosylcytosine.

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## SYNTEZA I WŁASNOŚCI O′-METYLO I O′-ETYLO POCHODNYCH 1-β-D-ARABINOFURANOZYLOCYTOZYNY I 1-β-D-ARABINOFURANOZYLOURACYLU


Streszczenie

1. Przez alkilowanie 1-β-D-arabinofuranozylocytozyny (araC) za pomocą siarczanu dwumetylu bądź siarczanu dwuetylu w środowisku zasadowym, a następnie chromatografię kolumnową na zasadowym jonicie uzyskano z dobrą wydajnością wszystkie *O′*-metylo i *O′*-etylo pochodne araC.

2. *O′*-Alkilowe analogi 1-β-D-arabinofuranozylouracylu (araU) otrzymano przez dezaminację odpowiednich analogów araC w 1 M-$CH_3COOH$; przedstawiono wstępne wyniki badań nad mechanizmem oraz kinetyką tej reakcji.

3. Identyfikację czternastu *O′*-alkilo pochodnych araC oparto głównie na widmach PMR oraz na szybkości dezaminacji w 1 M-$CH_3COOH$.

4. Na podstawie sekwencji elucji różnych *O′*-alkilo pochodnych na zasadowej kolumnie jonitowej określono względne kwasowości trzech hydroksyli. Kolejność elucji, wraz z wynikami widm u.v. w środowisku alkalicznym dla niektórych pochodnych, sugerują istnienie wiązania wodorowego między 2′-OH i 5′-OH.


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*Addendum* (added in proof): Since submission of the foregoing manuscript, we have carried out detailed p.m.r. analyses of various araC and araU derivatives in strongly alkaline medium (E. Darżynkiewicz, M. Remin, A. Dworak & D. Shugar, *Cancer Biochemistry Biophysics,* in press). The results demonstrate the formation, under these conditions, of an intramolecular 5′-OH···2′-O⁻ hydrogen bond, hence similar to that suggested on page 314 of the present manuscript. We have also succeeded in measuring directly, by spectral titration, the p*K* values for sugar hydroxyls; these results will be elsewhere presented (E. Darżynkiewicz, H. Sierakowski & Shugar, in preparation).

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# SEPARATION OF THE COMPLEMENTARY STRANDS OF DNA BY CENTRIFUGATION IN ALKALINE $Cs_2SO_4$ GRADIENT CONTAINING $Ag^+$ OR $Hg^{2+}$

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A homogeneous DNA fraction from mouse liver ("mouse satellite") was centrifuged in an alkaline $Cs_2SO_4$ gradient in the presence of silver or mercury ions. In both cases, the strand separation was better than that obtained in an alkaline CsCl density gradient. Conditions for strand separation in a $Cs_2SO_4$ - $Ag^+$ - $OH^-$ density gradient are discussed.

This work was undertaken to find means of separating the strands of DNAs which are not, or are barely, separable by standard alkaline CsCl centrifugation. This method, excellent for some purposes (for details see review by Flamm *et al.*, 1972) has one important limitation, namely it does not allow to change the densities of the strands, so the extent of the separation is imposed in advance. At the beginning, we added $HgCl_2$ to the alkaline CsCl gradient. In the range of low mercury to DNA ratios, the separation was worse than in the alkaline CsCl alone. Augmentation of this ratio brought about an increase in the buoyant density of the DNA-$Hg^{2+}$ complex to such an extent that it was greater than that obtained with the CsCl gradient. We then applied the $Cs_2SO_4$ gradient which is steeper and in which we could obtain a greater range of densities.

One of the homogeneous fractions of mouse liver DNA commonly known as "mouse satellite" was selected as a model since it gives good, but not complete, strand separation in a preparative alkaline CsCl gradient.

## MATERIALS AND METHODS

DNA from liver of mouse (inbred CDBA strain) was isolated by the method of Savitzky & Stand (1966).

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*Preparative* $Cs_2SO_4$ - $Ag^+$ *density gradient centrifugation.* To DNA dialysed exhaustively against 5 mM-$Na_2B_4O_7$ and adjusted to a concentration of 50 µg/ml was added solid $Cs_2SO_4$, suprapure (E. Merck, Darmstadt, G.F.R.) to obtain a density of 1.49 g/ml, and 0.05 mM-$AgNO_3$ to obtain a silver to nucleotide molar ratio, $r=0.3$. The sample, 25 ml, was placed in a nitrocellulose tube, then overlaid with liquid paraffin and centrifuged in a Spinco no. 30 rotor for 60 h at 27 000 r.p.m. and 20°C. The collected fractions were dialysed first against 1 M-NaCl and then against 0.01 M-NaCl - 0.01 M-Tris-HCl, pH 9.5, and used for preparative CsCl gradient centrifugation.

*Preparative* CsCl *density gradient centrifugation.* To DNA in 0.01 M-NaCl - 0.01 M-Tris-HCl, pH 9.5, adjusted to a concentration of 15 µg/ml, was added solid CsCl, suprapure (E. Merck) to obtain a density of 1.69 g/ml. The sample, 8.5 ml, was placed in a nitrocellulose tube, then overlaid with liquid paraffin and centrifuged in a Spinco no. 40 rotor for 42 h at 37 000 r.p.m. and 20°C. The collected fractions were dialysed against 5 mM-$Na_2B_4O_7$ or 0.01 M-NaCl.

*Preparative alkaline* $Cs_2SO_4$ *gradient centrifugation.* DNA in 5 mM-$Na_2B_4O_7$ was adjusted to the required volume and concentration. To the sample were added: solid $Cs_2SO_4$, 1 M-NaOH, 1% sodium dodecyl sulphate (SDS) and different amounts of $AgNO_3$, and the mixture centrifuged in a Spinco no. 40 rotor for 64 h at 37 000 r.p.m. and 20°C, or in a Spinco no. 50 rotor for 24 h at 42 000 r.p.m.

A typical mixture contained 6.7 ml of DNA in 5 mM-$Na_2B_4O_7$, 0.36 ml of 1 M-NaOH, 0.19 ml of 1% SDS, 6.05 g of $Cs_2SO_4$ and 0.013 ml of 5 mM-$AgNO_3$. In this case the total volume was 8.5 ml, density 1.564 g/ml, silver to nucleotide molar ratio, $r=0.2$, and DNA concentration 0.25 $E_{260}$ unit.

*Preparative alkaline* CsCl *gradient centrifugation.* To a mixture of 5.65 ml DNA dialysed against 0.01 M-NaCl, 0.36 ml of 1 M-NaOH and 0.19 ml of 1% SDS, was added 8.69 g of solid CsCl. Total volume was 8.5 ml, density $\rho=1.75$ g/ml, and DNA concentration 12 µg/ml. The sample was covered with liquid paraffin and centrifuged in a polyethylene tube in the no. 40 rotor of a Spinco L 50 ultracentrifuge for 40 h at 37 000 r.p.m. and 20°C.

*Analytical alkaline* $Cs_2SO_4$ *gradient centrifugation.* The sample was prepared by mixing 2 ml of DNA solution of 10 µg/ml and 0.057 ml of 1 M-NaOH. To 0.85 ml of the mixture, 0.05 ml of $AgNO_3$ or $H_2O$ was added, followed by solid $Cs_2SO_4$. The concentration of $AgNO_3$, and quantity of $Cs_2SO_4$ were calculated to obtain the desired silver to nucleotide ratio and density of solution. The sample was centrifuged in a Spinco model E ultracentrifuge for 20 h at 44 770 r.p.m. at 20°C.

*Analytical* CsCl *gradient centrifugation.* DNA, 5 µg, in 0.7 ml of 0.15 M-NaCl - 0.015 M-sodium citrate (SSC), to which enough solid CsCl was added to obtain a density of 1.700 g/ml, was centrifuged in a Spinco model E ultracentrifuge for 24 h at 44 770 r.p.m. and 25°C. Bacterial DNA with a buoyant density of 1.740 g/ml was used as a density marker.

*Analysis of the separated DNA fractions.* After density gradient preparative centrifugation, the fractions were collected by suction from the bottom of the tube, then pumped through a 0.3 cm cell of the LKB Uvicord spectrophotometer, and

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transmission at 253.7 nm recorded. In some experiments, $E_{260}$ and the refractive index of each fraction were measured.

*Melting curves* were obtained in a Beckman DU spectrophotometer equipped with a thermostated cell holder, heated at a rate of 1°C/min.

*Reassociation*. DNA from heavy band H, from light band L, and the mixture of both, was heated at 50°C for 24 h in 1 M-NaCl - 0.1 × SSC. DNA concentration was 20 μg/ml.

## RESULTS AND DISCUSSION

A homogeneous DNA fraction, commonly known as "mouse satellite", was obtained by $Cs_2SO_4$ gradient centrifugation with addition of silver nitrate according to Corneo *et al.* (1968). In the transmission profile (Fig. 1) two bands lighter than the main band are clearly visible. The lightest band was collected and centrifuged in a CsCl gradient, then rerun in an alkaline gradient of CsCl, and in an alkaline gradient of $Cs_2SO_4$ containing $AgNO_3$ or $HgCl_2$ (Fig. 2). It was checked spectrophotometrically that the presence of $Cl^-$ in the alkaline solution of $Cs_2SO_4$ did not disturb the formation of the $Hg^{2+}$-DNA complex. In all three experiments DNA was resolved into two bands. The bands obtained in alkaline CsCl corresponded to the complementary strands of DNA (Flamm *et al.*, 1967).

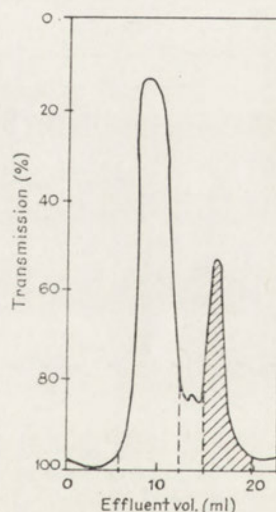


Fig. 1. Preparative density gradient fractionation of mouse DNA in $Cs_2SO_4$ with $AgNO_3$. Initial density 1.48 g/ml, $r = 0.3$, concentration 0.960 $E_{260}$ unit, 25 ml per tube, Spinco no. 30 rotor, 27 000 r.p.m., 60 h, 20°C. The hatched fraction was used for the next step of purification.

To see whether the two bands obtained in $Cs_2SO_4$ - $OH^-$ - metal ion gradient correspond to the complementary DNA strands, the melting profiles of the DNA from either band, and their mixture, reassociated for 24 h at 60°C in 1 M-NaCl - 0.1 × SSC, were examined. The melting profiles for DNA from single bands resembled that for non-reassociated DNA, whereas the profile of the mixture showed a relatively sharp transition and 28% hyperchromicity (Fig. 3). The results obtained with $Hg^{2+}$ or $Ag^+$ as complexing agent were virtually the same. This shows that under our experimental conditions both with mercury and silver ions the homogeneous DNA fraction separated into two complementary strands.

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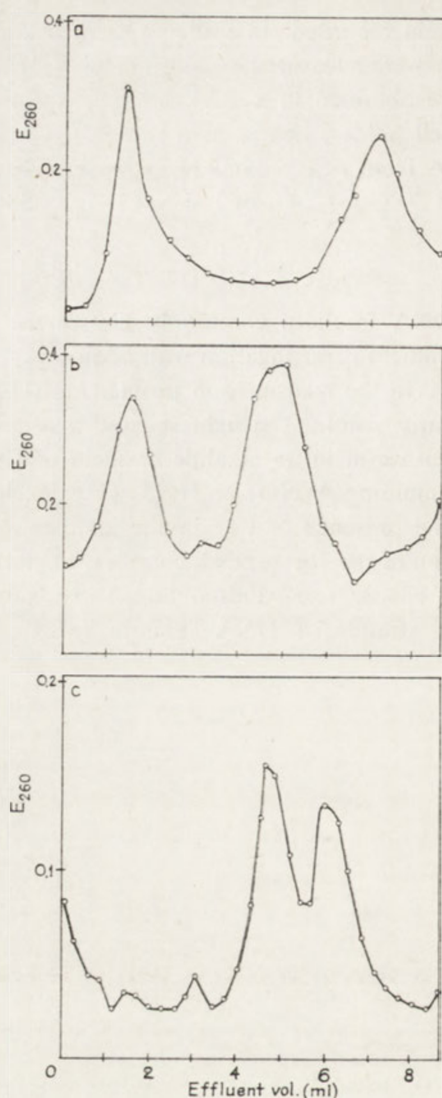


Fig. 2. Strand separation by preparative ultracentrifugation: *a*, alkaline $Cs_2SO_4$ with $Ag^+$, $r=0.3$, initial density 1.55 g/ml; *b*, alkaline $Cs_2SO_4$ with $Hg^{2+}$, $r=0.3$, initial density 1.55 g/ml; *c*, alkaline CsCl, initial density 1.75 g/ml. Spinco no. 40 rotor, 37 000 r.p.m., 40 h, 20°C.

Further experiments aimed at characterizing the separation of DNA strands were done in an alkaline $Cs_2SO_4$ gradient containing silver salt.

In the absence of metal ions in alkaline solutions at pH$\geqslant$12.5, mouse homogeneous DNA fractions centrifuged in the Spinco no. 40 rotor gave two barely resolved bands with densities of 1.448 g/ml and 1.432 g/ml, which correspond to the densities of heavy (H) and light (L) strands, 1.448 g/ml and 1.434 g/ml, respectively, as calculated from the literature data. Calculation was based on the assumption that the buoyant density of polynucleotide, $\rho=\Sigma\rho_i\,\mu_i$, where $\rho_i$ is the buoyant

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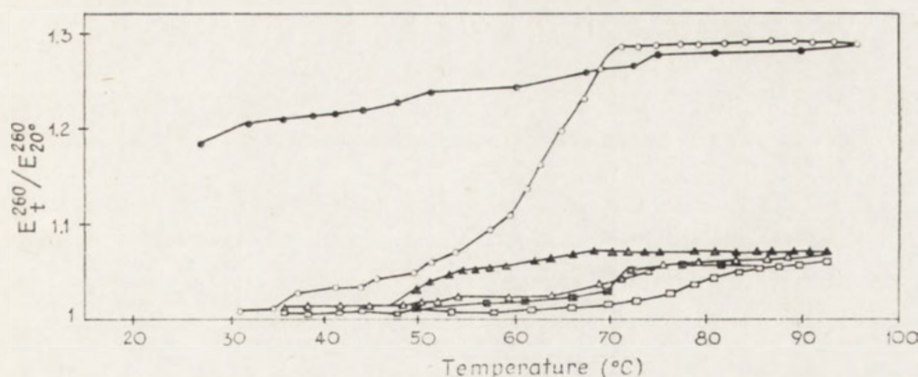


Fig. 3. Thermal denaturation of DNA from the heavy (H) and light (L) bands obtained after alkaline $Cs_2SO_4$ - $Ag^+$ gradient centrifugation (cf. Fig. 2a), and denaturation of the mixture of the two strands. Fraction H: □, heating; ■, cooling. Fraction L: △, heating; ▲, cooling. Mixture H+L: ○, heating; ●, cooling. The fractions were preincubated for 24 h at 60°C in 1 M-NaCl - 0.1×SSC.

density of homopolydeoxyribonucleotide *i* in alkaline $Cs_2SO_4$, and $\mu_i$, the molar ratio of nucleotide *i* in the single-stranded polynucleotide. Numerical data are presented in Table 1. Data for base composition are from Corneo *et al.* (1968) and for densities of homopolydeoxyribonucleotides from Wells & Larson (1972).

Table 1

*Base composition of separated L and H strands and buoyant density data for homopolydeoxyribonucleotides*

Calculations based on the data of Corneo *et al.* (1968) and Wells & Larson (1972).

| $i$ | L strand ($\mu_i$) | H strand ($\mu_i$) | pH 12.5, $Cs_2SO_4$, homopolydeoxyribo $i$ ($\rho_i$) |
|---|---|---|---|
| A | 0.448 | 0.192 | 1.380 |
| T | 0.202 | 0.458 | 1.447 |
| G | 0.220 | 0.140 | 1.540 |
| C | 0.130 | 0.214 | 1.422 |

Analytical centrifugation of "mouse satellite" DNA gave a single peak in $Cs_2SO_4$ at pH$\geqslant$12.5. The band density was 1.437 g/ml. Addition of silver nitrate to the alkaline $Cs_2SO_4$ - DNA solution induced a considerable increase in the density of the heavy strand and a slight increase of the light one, as observed by preparative centrifugation (Fig. 4). This increase depended on the amount of silver nitrate added. Scattering of the experimental points could be accounted for by the use of four different DNA preparations.

In addition it appeared that the DNA concentration was of importance for the equilibrium of complex formation. Comparing the data for the density of the separated strands from analytical and preparative centrifugation we found that the densities determined analytically were greater than those determined preparatively

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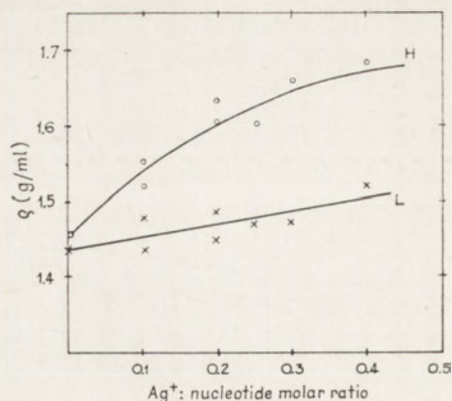


Fig. 4. Influence of silver to nucleotide ratio on the densities of complementary strands in alkaline $Cs_2SO_4$ gradient. H, heavy strand; L, light strand.

even for the same DNA preparation and the same silver to nucleotide ratio. When this ratio exceeded $r=0.1$, only a single band was observed (Fig. 5). It seems that the heaviest strand became so heavy that it could not be visualized in analytical runs.

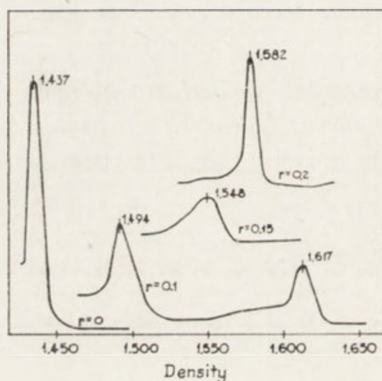


Fig. 5. Analytical $Cs_2SO_4$ density gradient centrifugations of purified "mouse satellite" DNA. Numbers indicate silver to nucleotide ratio $r$ and buoyant density.

It may be supposed that the equilibrium in the system DNA, $Ag^+$, $OH^-$, DNA - $Ag^+$ or hypothetical DNA - AgOH complex is sensitive to changes in the concentrations of the components of the system.

The buoyant densities in neutral CsCl of the DNA strands separated by $Cs_2SO_4$ - $Ag^+$ - $OH^-$ gradient centrifugations were 1.727 g/ml for the heavy strand and 1.697 for the light strand, so that the relative position of each strand in this gradient was the same as in an alkaline CsCl gradient. The strand separation in the CsCl alkaline gradient is believed to be connected with the dissociation of the thymidine and guanosine in the alkaline solution, and with substitution of the $H^+$ ion by the

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heavy $Cs^+$ ion. The differences in the densities of the strands are related to the differences in G-T contents. Such relation is not observed in an alkaline $Cs_2SO_4$ gradient (Wells & Larson, 1972). Additional difficulty in the interpretation arises from the fact that the silver ion is lighter than the caesium ion.

In any case we believe that the method provides new possibilities for isolation of homogeneous DNA fractions from the genome of higher organisms. This procedure also improves strand separation of the DNA as compared to an alkaline CsCl gradient.

In preliminary experiments with a guinea pig DNA homogeneous fraction (known as "α satellite", Flamm *et al.*, 1968), we have obtained similar results.

Some years ago Gruenwedel & Davidson (1967) observed splitting of the peak of phage DNA centrifuged in alkaline $Cs_2SO_4$ - methyl - mercury hydroxide gradient. In the light of the results presented here it seems that these authors achieved real strand separation.

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We wish to express our cordial thanks to the staff of the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, and especially to Dr. J. Šponar and Dr. H. Vatavova for their discussion, help in analytical centrifugation experiments and hospitality during the stay of one of us (J. F.) in Prague. Skilfull technical assistance of Mrs. K. Chorąży is acknowledged.

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## ROZDZIAŁ KOMPLEMENTARNYCH WSTĘG DNA PRZEZ ULTRAWIROWANIE W ALKALICZNYM ROZTWORZE $Cs_2SO_4$ - $Ag^+$ ($Hg^{2+}$)

### Streszczenie

Homogenną frakcję DNA otrzymanego z wątroby myszy (mysi „satelitarny" DNA) rozdzielaliśmy na nici komplementarne przez ultrawirowanie w alkalicznym roztworze $Cs_2SO_4$ w obecności jonów srebra i rtęci. W obydwu przypadkach rozdział był lepszy niż rozdział uzyskany w alkalicznym roztworze CsCl drogą ultrawirowania. W pracy dyskutujemy warunki rozdziału na nici komplementarne w układzie $Cs_2SO_4$ - $Ag^+$ - $OH^-$.

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# THE ACTIVITY OF ATP CITRATE LYASE IN RAT BRAIN AND LIVER UNDER VARIOUS PHYSIOLOGICAL CONDITIONS*

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1. The activity of ATP citrate lyase in brain was about 35% higher in newborn rats than in adult animals; in the latter animals it was not affected by diet or diabetes. 2. The brain and liver enzyme showed the same basic kinetics, i.e. pH optima (7.8), $K_m$ values (for ATP 0.42 mM and citrate $K_{m_1}$ 0.13, $K_{m_2}$ 2.3 mM) and $K_i$ for ADP (0.20 mM). This holds both for newborn and adult rats, starved or fed a standard or carbohydrate diet. 3. α-Oxoglutarate inhibited by about 50% both the brain and liver enzymes, irrespective of the age of the animals. 4. The role of citrate lyase in lipogenesis in brain is discussed.

ATP citrate lyase (EC 4.1.3.8) is a cytoplasmic enzyme involved in displacement of acetyl-CoA groups from mitochondria to cytoplasm (Srere, 1965; Daikuhara *et al.*, 1968). It has been demonstrated that the activity of this enzyme increases under conditions favouring biosynthesis of fatty acids in liver, adipose tissue and mammary gland, and is decreased upon lowering of lipogenesis (Ballard & Hanson, 1967; Angielski & Szutowicz, 1967; Kornacker & Lowenstein, 1965a,b; Smith & Abraham, 1970). The regulatory role of this enzyme is, however, controversial (Foster & Srere, 1968; Goodridge, 1968).

In the nervous tissue, the severalfold decrease in fatty acid biosynthesis observed during development was accompanied by a 40% only decrease in ATP citrate lyase activity (D'Adamo & D'Adamo, 1968). This lack of parallelity may be due to participation of other sources of acetyl-CoA such as acetoacetate and acetoaspartate (D'Adamo *et al.*, 1968; Buckley & Williamson, 1973), changes in concentration of low-molecular-weight effectors, or kinetic properties of this enzyme during development of the animal.

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Therefore it seemed of interest to examine this last supposition and compare kinetic properties of ATP citrate lyase obtained from nervous tissue showing different lipogenic activity.

## MATERIALS AND METHODS

*Reagents.* CoA, NADH, ATP, *cis*- and *trans*-aconitate, DL- and D-*threo*-isocitrate were from Sigma Chem. Comp. (St. Louis, Mo., U.S.A.), Sephadex G-200 from Pharmacia (Uppsala, Sweden), dithiothreitol and mercaptoethanol from Calbiochem AG (Lucerne, Switzerland), insulin from Novo (Copenhagen, Denmark); other reagents were from P.O.Ch. (Gliwice, Poland). CoA was standardized for CoA-SH content using ATP citrate lyase from rat liver (Szutowicz *et al.*, 1972).

*Animals.* White Wistar rats of either sex were used. Young animals were removed from their mothers on the 30-th day of life. Adult animals (3 - 4 months old, weighing 180 - 250 g) were subjected to the following alternative treatments: *a*) fed standard diet (Drug Institute, Warsaw, Poland); *b*), fasted for 72 h; *c*) fasted for 48 h, then refed white wheat bread; *d*) treated as under *c* and injected intraperitoneally with actinomycin D (0.25 mg/kg body wt. per day), the first dose being given 1 h before refeeding; *e*) rats with induced diabetes (blood glucose concentration of 400 - 500 mg%): the animals were fasted for 24 h, given a single intraperitoneal dose of alloxan (0.14 g/kg body wt.) and fed standard diet; beginning from the third day of the experiment, they were injected subcutaneously every day with the insulin solvent for another 3 days; *f*) diabetic rats, treated for 3 days as in *e*, and beginning from the third day given daily for 3 days 4 units of insulin (mixture of crystalline and ultralente, 1:1) per day; the animals were killed 24 h after the last injection.

All manipulations on the animals were performed at 9 - 10 a.m.

*Preparation of ATP citrate lyase.* Brains and livers were homogenized with 2 and 7 vol., respectively, of cold 0.2 M-KCl in 5 mM-Tris-HCl buffer, pH 7.4, containing 1 mM-dithiothreitol, in an all-glass homogenizer with a teflon pestle (600 rev./min). The homogenates were centrifuged at 30 000 *g* for 40 min at 0 - 4°C and the obtained supernatant was used as a crude enzyme.

*Purification of ATP citrate lyase.* A 40-fold purification of the enzyme from adult rat brain, and 10 - 20-fold purification from the brain of 1 - 2-day-old animals, were obtained by repeated fractionation with ammonium sulphate and Sephadex G-200 gel filtration as described elsewhere (Szutowicz *et al.*, 1974). The enzyme from liver was purified by the same procedure with minor modifications, the obtained degree of purification being 30 - 50-fold in the case of rats fasted for 72 h, and 10 - 15-fold from those on carbohydrate diet.

The obtained preparations of ATP citrate lyase were devoid of oxoglutarate dehydrogenase (EC 1.2.4.2), isocitrate dehydrogenase (NAD and NADH) (EC 1.1.1.41 and 1.1.1.42) or L-lactate dehydrogenase (EC 1.1.1.27). They contained, however, traces (0.5%) of aconitate hydratase (EC 4.2.1.3) and up to 3% of malate dehydrogenase (EC 1.1.1.37).

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*The assay of ATP citrate lyase.* The enzyme activity was determined by measuring the amount of oxaloacetate formed, using malate dehydrogenase and NADH. The standard incubation mixture contained in a final volume of 0.5 ml: 50 mM-Tris-HCl buffer, pH 7.8; 5 mM-ATP, 20 mM-potassium citrate, 10 mM-$MgCl_2$, 10 mM-2-mercaptoethanol, 100 mM-KCl, 0.2 mM-CoA, 0.15 mM-NADH, 2 i.u. of malate dehydrogenase and the enzyme preparation studied. The samples containing no CoA were preincubated with the enzyme for 5 min at 37°C, and the reaction was started by adding CoA. After 10 min incubation at the same temperature, the reaction was stopped by adding 0.7 ml of 0.2 M-NaOH containing 0.08 M-$KH_2PO_4$, and the sample placed in refrigerator for 6 h. The sediment formed was centrifuged off, and the extinction at 340 nm was measured in the clear supernatant. The rate of the reaction was linear for 15 min at the protein and substrate concentrations used.

*Determination of protein concentration.* Protein was determined by the biuret method (Gornall *et al.*, 1949) using bovine serum albumin (fraction V) as a standard; 0.5 ml of the biuret reagent gave with a 0.1 ml sample (0.5 mg of protein) the $E_{546}$ value of 0.4 (light-path 2 cm).

## RESULTS

The activity of ATP citrate lyase per 1 g of brain tissue was by about 35% higher in newborn rats than in adult animals, whereas no significant differences were observed in the activity of this enzyme from brain of young (10 - 30 days old) or adult animals (Table 1). It was found that carbohydrate diet, starvation, diabetes or insulin administration had no effect upon this enzymic activity in adult animals.

In liver of young, suckling rats the activity of ATP citrate lyase decreased to values lower by a factor of 3 - 6 as compared with those found during the first two days of life. On weaning of young animals to standard diet, the enzyme activity increased to values about twice as high as at birth. A 72-h fast lowered this activity by a half, and induced diabetes resulted in a 40% decrease in enzyme activity as compared with rats kept on standard diet. On the other hand, in animals fed for 72 h a high-carbohydrate diet, a considerable increase in the activity of citrate lyase was observed. This increase could be prevented by administration of actinomycin D.

The optimum pH estimated for the purified enzyme from the brain of newborn and adult rat, was found to be 7.8 (Fig. 1), and was the same as that for liver enzyme (Szutowicz *et al.*, 1971). No differences were also found in $K_m$ values (0.42 mM) with ATP, as compared with liver enzyme.

At a stable concentration of ATP (5 mM) and $MgCl_2$ (10 mM), the Lineweaver-Burk plot for citrate had a biphasic character, as it was shown previously for the liver enzyme (Szutowicz & Angielski, 1970). Consequently, two values of $K_m$ were estimated (Table 2); $K_{m_1}$ corresponding to low citrate concentration was 0.12 mM for the brain enzyme from newborn rats and 0.14 mM from adult rats. For the liver enzyme from adult rats, both those fasted for 72 h or kept for the same period

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## Table 1

*Effect of age and nutrition on the activity of ATP citrate lyase in rat brain and liver*

The results are mean values ± S.E.M., from the number of experiments indicated in parentheses.

| Animals, age and treatment | Brain | | Liver | |
|---|---|---|---|---|
| | mmol/mg protein/min | mmol/g tissue/min | mmol/mg protein/min | mmol/g tissue/min |
| Young, kept with mothers: | | | | |
| 0 - 2 days old | 5.7±0.9 (3) | 152±11 | 18.7± 5.2 (3) | 807±80 |
| 10 - 14 days old | 3.8±0.3 (5) | 96±9[a] | 2.9± 0.6[a] (5) | 145±79[a] |
| 21 - 30 days old | 4.3±1.0 (4) | 107±16 | 5.0± 1.9[a] (4) | 537±184 |
| Adult: | | | | |
| standard diet | 4.5±0.4 (12) | 99±5[a] | 35.9± 2.6[a] (12) | 2240±295[a] |
| fasting 72 h | 4.2±0.2 (5) | 87±9 | 15.0± 2.0[b] (5) | 1090±80 |
| fasting 48 h+ carbohydrate diet 72 h | — | — | 198.0±32.0[b] (5) | 13500±2000 |
| fasting 48 h+ carbohydrate diet 72 h+actinomycin D (0.25 mg/kg body wt.) | — | — | 17.5± 4.9[b] (7) | 1540±510 |
| diabetic | 4.4±0.4 (6) | 101±3 | 19.5± 3.5[b] (7) | 1310±215 |
| diabetic treated with insulin (4 u., 3 days) | 4.4±0.2 (5) | 92±7 | 57.3±10.4[c] (5) | 3630±630[c] |
| standard diet+ insulin (4 u., 3 days) | 4.6±0.4 (8) | | 54.0± 2.8[b] (8) | |

[a] Difference statistically significant in relation to animals 0 - 2 days old ($0.005 < P < 0.05$).
[b] Difference statistically significant in relation to animals fed standard diet ($0.001 < P < 0.025$).
[c] Difference statistically significant in relation to diabetic animals ($0.01 < P < 0.05$).

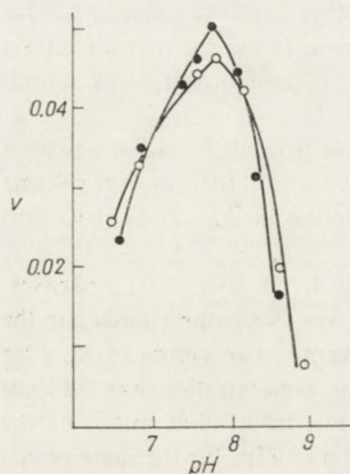


Fig. 1. Effect of pH on the activity of ATP citrate lyase from the brain of: (●), 0 - 2-day-old and (○), adult rats. The reaction mixture contained in 0.5 ml: 2 mM-citrate, 1 mM-ATP, 4 mM-$MgCl_2$, and enzymic protein, 0.05 mg. Other components of the mixture and the assay of activity were as described under Materials and Methods.

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of time on carbohydrate diet, $K_{m_1}$ was 0.11 mM. The $K_{m_2}$ values, corresponding to high citrate concentrations, were also similar for all the preparations studied and ranged, respectively, from 2.38 to 2.12 mM.

Table 2

*$K_m$ values for ATP citrate lyase from rat brain and liver*

$K_m$ values are expressed as mM.

| Tissue | Animals, age and treatment | Citrate $K_{m_1}$ | Citrate $K_{m_2}$ | ATP $K_m$ | CoA $K_m$ |
|---|---|---|---|---|---|
| Brain | 0 - 2 days old | 0.12 | 2.38 | 0.42 | — |
| | adult, standard diet | 0.14 | 2.32 | 0.42 | 0.0007 |
| Liver | adult | | | | |
| | fasting 72 h | 0.11 | 2.12 | — | — |
| | fasting 48 h+carbohydrate diet 72 h | 0.11 | 2.12 | — | — |
| | standard diet* | 0.13 | 5.30 | 0.45 | — |

* Data from Szutowicz & Angielski (1970).

$K_i$ value for ADP, a competitive inhibitor of ATP citrate lyase (Inoue *et al.*, 1966; Szutowicz & Angielski, 1970) was the same with the brain enzyme of both adult and newborn rats, 0.19 and 0.16 mM, respectively (Fig. 2).

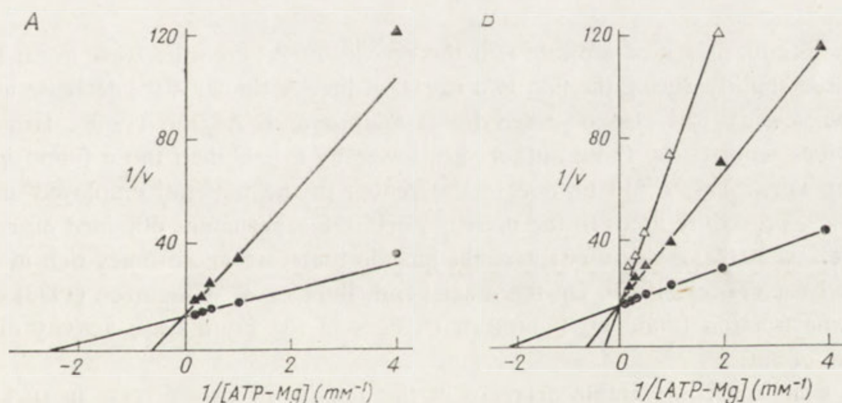


Fig. 2. Lineweaver-Burk plots for the effect of ADP on the ATP dependence of the activity of brain ATP citrate lyase from: *A*, 0 - 2-day-old and B, adult rats. (●), Control; (▲), with 0.5 mM-ADP-Mg; (△), with 1.0 mM-ADP-Mg. Both ADP and ATP were added at equimolar concentration with $MgCl_2$. Enzyme assay as under Methods. Protein, 0.05 mg.

As it may be seen in Table 3, α-oxoglutarate and pyruvate inhibited ATP citrate lyase from rat brain in 50 and 20%, respectively, at both citrate concentrations used. The 15 - 25% inhibition of the enzyme by aconitate and isocitrate was abolished on increasing citrate concentration. Other organic acids studied: succinate, fumarate and L-malate, had no effect on the activity of the brain enzyme.

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Table 3

*Effect of organic acids on the activity of ATP citrate lyase in brain of adult rats*

The reaction mixture was as described under Methods, except that the concentration of citrate was varied, as indicated. The compounds studied were added at concentration of 10 mM. Protein, 0.06 mg and 0.03 mg/sample at citrate concentrations of 0.5 mM and 20 mM, respectively. Inhibition (—) or activation (+) are expressed as percentages in relation to the activity of the control sample.

| Addition | 0.5 mM-citrate | 20 mM-citrate |
|---|---|---|
| None, control | 0 | 0 |
| DL-Isocitrate | —15 | +8 |
| *cis*-Aconitate | —24 | +3 |
| α-Oxoglutarate | —46 | —46 |
| Pyruvate | —19 | —20 |
| Succinate | —6 | —4 |
| Fumarate | —5 | — |
| L-Malate | —1 | 0 |

Oxoglutarate exerted practically the same inhibitory effect (50%) with the preparations obtained from brain of newborn and adult rats, and from liver of animals fasted or kept on carbohydrate diet.

## DISCUSSION

The results presented indicate that the activity of ATP citrate lyase in rat brain decreased slightly during the first two weeks of life. A similar 40% decrease in this enzyme activity was also observed by D'Adamo & D'Adamo (1968). However, the values reported by those authors are lower by a half than those found in the present work. This could be due to differences in the material employed and/or analytical procedure used. In the present work, the supernatant obtained on centrifugation at 30 000 *g* was used; it could include therefore microsomes rich in ATP citrate lyase (Tuček, 1967). On the other hand, Buckley & Williamson (1973) using the same fraction found no significant changes in the brain lyase activity during growth of rat.

In liver, the considerable decrease in the activity of citrate lyase in sucklings could be due to feeding on milk which contains non-saturated long-chain fatty acids. This observation is similar to that of Smith & Abraham (1970) for mouse liver, in which at the same time biosynthesis of fatty acids from precursors of citrate was lowered.

The observed parallelity between ATP citrate lyase activity and fatty acid synthesis in liver of adult rats, in response to changes in diet or induced diabetes, is in agreement with the results of Kornacker & Lowenstein (1965a,b) and Ballard & Hanson (1967).

The rate of acetyl-CoA formation by ATP citrate lyase could be also regulated, both in brain and liver, by changes in kinetic properties of the enzyme. This pheno-

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menon was observed by Volpe & Kishimoto (1972) and Land & Clark (1973) with respect to brain fatty acid synthetase during growth of rats.

No such differences in kinetic properties of citrate lyase were, however, found in our experiments. The enzyme showed the same biphasic course of reaction, with similar $K_{m_1}$ and $K_{m_2}$ values for preparations from the brain of adult and new-born rats, and from liver of starved or carbohydrate-fed animals. This could suggest the occurrence of two molecular forms of this enzyme, showing, respectively, high and low activity toward citrate (Plowman & Cleland, 1967; Szutowicz & Angielski, 1970).

The $K_i$ values for ADP were found to be lower by a half than the physiological concentration of this metabolite in brain (Folbergrova *et al.*, 1972a), which seems to suggest that the enzyme *in vivo* could be inhibited to a high extent both in newborn and adult animals. On the other hand, the ATP/ADP ratio in brain is about 10 (Granholm *et al.*, 1969); therefore the above conclusion should be taken with caution.

It seems that inhibition by α-oxoglutarate has no significance for the ATP citrate lyase activity *in vivo* (Folbergrova *et al.*, 1972b); the $K_i$ value for this compound, 9.0 mM (Szutowicz *et al.*, 1974) is severalfold higher than its concentration in brain, and is independent of the physiological state of the animal. It is of interest that α-oxoglutarate inhibits *in vitro* bacterial citrate lyase (EC 4.1.3.6) and citrate synthase (EC 4.1.3.7) (Michal & Bergmeyer, 1965; Srere, 1968). This could be considered as an additional evidence for the common phylogenetic origin of these three enzymes, postulated by Srere (1968).

The effect of ADP and α-oxoglutarate on ATP citrate lyase was practically independent of the age of the animals and the extent of lipogenesis, both with respect to the brain enzyme which depends only to a limited degree on the physiological state of the animal, and the liver enzyme the activity of which changes with the diet.

Lack of response of the brain ATP citrate lyase to the changes in lipogenesis is in disagreement with the assumed regulatory role of this enzyme on lipogenesis.

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## AKTYWNOŚĆ LIAZY CYTRYNIANOWEJ ATP-ZALEŻNEJ Z MÓZGU I WĄTROBY SZCZURA W RÓŻNYCH WARUNKACH FIZJOLOGICZNYCH

### Streszczenie

1. Aktywność liazy cytrynianowej w mózgu szczurów nowonarodzonych była o 35% wyższa niż u zwierząt dorosłych; zmiana diety, jak również cukrzyca były bez wpływu na aktywność liazy mózgowej zwierząt dorosłych.

2. Preparaty liazy uzyskane z mózgu i wątroby wykazują takie same własności kinetyczne, tj. optimum pH (7,8), wartości $K_m$ (dla ATP 0,42 mM i dla cytrynianu $K_{m_1}$ 0,13 mM i $K_{m_2}$ 2,3 mM) oraz wartość $K_i$ dla ADP (0,20 mM). Podobne wartości uzyskano dla szczurów nowonarodzonych, jak i dorosłych — niezależnie od stosowanej diety.

3. α-Ketoglutaran hamuje w około 50% aktywność enzymu pochodzącego z mózgu i wątroby szczura, niezależnie od wieku badanych zwierząt.

4. Przedyskutowano rolę liazy cytrynianowej w lipogenezie tkanki mózgowej.

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# POLYRIBOSOME FORMATION AND RNA SYNTHESIS AFTER BREAKING THE DORMANCY OF SUGAR BEET ROOT

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1. Breaking the dormancy of sugar beet root by slicing results in an instantaneous association of preexisting ribosomes into polyribosome structures; the maximum level of polyribosomes (66%) is attained after 6 h ageing of the tissue, and remains nearly constant thereafter. 2. Initiation of RNA synthesis is observed already after 1 h ageing but the early polyribosome development does not rely exclusively on supply of new RNA, since formation of polyribosomes occurs also in the absence of RNA synthesis. It is suggested that preexisting mRNA is used for this early polyribosome formation. 3. RNA synthesis is renewed after breaking tissue dormancy with preferential synthesis of mRNA, while initiation of rRNA synthesis occurs at the later stage. 4. Ordered derepression of genome activity proceeds with increasing formation of certain RNA species not entering polyribosomal complex; this species might represent population of the specific RNA, strictly confined to the nucleus.

Dormant state of roots and tuberous storage organs can be easily broken by cutting the tissue into small segments and placing them under suitable temperature conditions. This simple act of slicing induces a wide spectrum of metabolic changes which lead to a new biochemical state of the plant tissue. It has been shown by Click & Hackett (1963) that the dramatic increase in the rate of respiration following excision of potato tubers could be totally prevented by inhibitors of RNA and protein synthesis. This strongly suggested that gene derepression was a prerequisite for transition from the dormant to the active metabolic state.

Leaver & Key (1967) observed that polyribosome formation during ageing of the sliced carrot root tissue depended on RNA synthesis. This conclusion was next confirmed in similar experiments with potato tuber discs (Kahl, 1971).

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In the present work, we have attempted to see whether the preexisting RNA or thn ewley made RNA was responsible for the instantaneous polyribosome formation observed after sugar beet tissue excision (Cherry, 1968). Establishment of the sequence of the synthesis of particular classes of cellular RNA in response to breaking dormancy of sugar beet root, was another goal of the present work.

## MATERIALS AND METHODS

*Chemicals.* The following chemicals were used: [5-$^{3}$H]Uridine (spec. act. 8 Ci/mmol) and $^{14}$C-reconstituted protein hydrolysate, 1 mCi/ml (Schwarz Mann Bioresearch, Orangeburg, N.Y., U.S.A.); [$^{32}$P]orthophosphoric acid, carrier-free (New England Nuclear Corporation, Boston, Mass., U.S.A., and the Radiochemical Centre, Amersham, England); actinomycin D (Dactinomycin) (Merck, Sharp and Dohme, Research Laboratory, Rahway, N.Y., U.S.A.); 5-fluorouracil (Hoffman LaRoche, Nutley, N.J., U.S.A.); RNase from bovine pancreas (Calbiochem, Los Angeles, Calif., U.S.A.); DNase (RNase-free) (Worthington Biochemical Corporation, Freehold, N.Y., U.S.A.); sucrose, ultra pure (special density gradient grade) (Schwarz Mann Co.); deoxycholic acid, sodium salt (DOC) and spermidine phosphate dihydrate (Sigma Chemical Company, St. Louis, Mo., U.S.A.); dextran (mol. wt. 60 000 - 90 000) (Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.).

*Plant material.* Sugar beets (*Beta vulgaris* L) were grown in the field and stored after harvest at 4°C. Both fresh and aged tissue was used in the course of the work. In the experiments involving ageing of sliced tissue, transverse sections of sugar beet root were cut into $2 \times 2$ mm squares, 1 mm thick and placed in sterilized Erlenmeyer flasks (under a sterile hood). Sliced tissue was repeatedly rinsed with sterile distilled water, and the sterile medium (1:1.5, v/w) containing 0.01 M-Tris-HCl, pH 6.5, and 0.005 M-$\beta$-mercaptoethanol was added. The flasks were shaken in a water bath at 30°C for the desired periods of time, the aeration medium being changed at least every 4 h. For RNA labelling studies, [$^{32}$P]phosphate or [5-$^{3}$H]uridine was introduced at 1, 2 or 6 h before the incubation was completed; when protein synthesis was investigated, $^{14}$C-reconstituted protein hydrolysate was added alternatively. In some experiments, actinomycin D (20 μg/ml) and 5-fluorouracil ($10^{-3}$ M) were added to the incubation medium. After incubation, the tissue was rinsed several times with cold 0.01 M-phosphate buffer or 1.0 mM-uridine to exchange the remaining isotope and then rinsed extensively with distilled water. Finally, the tissue was blotted dry on absorbent paper and immediately used for polyribosome and RNA isolation.

*Preparation and analysis of polyribosomes.* Samples of sugar beet root slices, 5 - 15 g, were rapidly ground in 2 vol. of prechilled medium containing 0.25 M-sucrose, 0.05 M-Tris-succinate, pH 7.8, 0.015 M-KCl, 0.01 M-$MgCl_2$, and 0.005 M-$\beta$-mercaptoethanol. At the end of grinding, freshly prepared 10% DOC was added to a final concentration of 1%. The resultant pulp was quickly filtered through a double layer of cheese cloth and the homogenate was centrifuged at 0 - 2°C using

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the SS-34 rotor of the Sorvall centrifuge at 10 000 *g* for 10 min. The supernatant was filtered through glass wool and then layered over a two-step sucrose gradient of 0.5 and 1.8 M-sucrose solutions, containing 0.01 M-Tris-succinate, pH 7.8, 0.015 M-KCl, 0.01 M-$MgCl_2$ (TKM buffer) and 0.005 M-$\beta$-mercaptoethanol, and centrifuged at 0 - 2°C in the Ti 50 rotor of the Beckman model L ultracentrifuge at 47 500 r.p.m. for 16 h. The resultant opalescent pellet was gently surface-washed with TKM buffer containing 0.005 M-$\beta$-mercaptoethanol, and then resuspended in a small volume of the same buffer (usually 0.5 ml per pellet).

Although the pelleted material contained both poly- and monoribosomes, for the sake of simplicity it will be further referred to as the polyribosomal complex.

Polyribosome suspension was transferred to a glass Dounce homogenizer and gently homogenized (3 - 4 strokes). An aliquot of suspended polyribosomal material (0.5 - 1.0 ml) was layered over a linear continuous 12 - 36% sucrose gradient containing 0.01 M-Tris-succinate, pH 7.8, 0.01 M-$MgCl_2$ and 0.001 M-spermidine, and centrifuged at 0 - 2°C in SW-25.1 rotor of Beckman ultracentrifuge at 24 500 r.p.m. for 3 h. All manipulations during polyribosome isolation and analysis were performed at 0 - 4°C, and all solutions used for polyribosome isolation and analysis were prepared with autoclaved distilled water.

The distribution pattern of ribosomes was recorded at 254 nm using the ISCO density gradient fractionator. Forty 0.6 ml fractions were collected. For radioactivity measurements each fraction was precipitated with an equal volume of 10% trichloroacetic acid containing 100 µg/ml of herring sperm DNA. After 30 min standing in the cold, the precipitates were collected on glass fiber filters (Whatman), washed repeatedly with 5% trichloroacetic acid, then allowed to dry, placed in scintillation fluid and the radioactivity determined in a Packard Tricarb liquid scintillation spectrometer.

*Release of RNA from polyribosomes and digestion with RNase.* Three various procedures for obtaining RNA from polyribosomes were compared: treatment with a) 10 mM-EDTA or b) 5 mM-Na-pyrophosphate, both causing dissociation of ribosomes into subunits accompanied by the release of mRNA and tRNA fractions, and c) 0.5% SDS - 10 mM-EDTA treatment which results in releasing of all three classes of RNA, rRNA included. The last procedure was found to be the most convenient, as it allowed for simultaneous estimation of the contribution of individual RNA fractions to the radioactivity of polyribosomal complex. The polyribosome suspension in TKM buffer was then treated with an equal volume of 0.01 M-Tris-succinate buffer, pH 7.8, containing 0.2 M-NaCl, 0.02 M-EDTA and 1% SDS. The sample was vigorously stirred for 10 min and the RNA released from polyribosomes was analysed in 5 - 25% sucrose gradient containing 0.01 M-Tris-HCl, pH 7.2, 0.1 M-NaCl and 0.005 M-EDTA, and centrifuged in an SW-25.1 rotor of the Beckman ultracentrifuge at 23 000 r.p.m. for 16 h. The absorbancy and radioactivity were determined as described for polyribosome preparations.

For controlled RNase digestion, polyribosome suspension in TKM buffer was treated either with 0.1 or 2 µg of pancreatic RNase per 1 ml for 10 min at room

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temperature, and the sample was directly layered over 12 - 36% linear sucrose gradient as described for the analysis of polyribosomes.

*Isolation and analysis of total RNA.* RNA was isolated from fresh and aged sugar beet root tissue by the standard phenol-SDS method (Cherry & Chroboczek, 1966). The preparation of total RNA was subsequently fractionated by layering over 5 - 25% linear sucrose gradient and centrifuging, as described for the RNA released from polyribosomes.

*Isolation of nuclei and extraction of nuclear RNA.* Nuclei from sugar beet root tissue were isolated as described by Rho & Chipchase (1962) using small "pea popper" (0.011 inch clearance). The crude nuclear pellet was resuspended in 0.25 M-sucrose containing 0.006 M-Tris-HCl, pH 7.2, and 0.003 M-$CaCl_2$, and the nuclei were further purified by centrifugation through a solution containing 18% dextran (mol.wt. 60 000 - 90 000) in 0.25 M-sucrose and 0.006 M-Tris-HCl, pH 7.2. The nuclei suspension was layered over the dextran solution and centrifuged for 30 min at 8000 r.p.m. in SW-25.1 rotor of the Beckman ultracentrifuge. RNA was extracted from the purified nuclei by the hot phenol-SDS technique as described by Girard (1967). The final preparation of nuclear RNA was dissolved in 0.01 M-Tris-HCl buffer, pH 7.2, containing 0.001 M-$MgCl_2$, and submitted to digestion with DNase (RNase-free, 20 μg/ml) for 20 min at 4°C, followed by removal of the enzyme by SDS-phenol extraction. RNA was recovered from aqueous phase by precipitation with 3 vol. of 95% ethanol and the purified nuclear RNA preparation was submitted to 5 - 25% sucrose gradient centrifugation under conditions described for total RNA.

The yield of the purified nuclei preparation was calculated on the basis of DNA content determined by the diphenylamine method (Burton, 1956) in the initial tissue homogenate and in the final preparation of nuclei. The average yield was 12.6%.

## RESULTS

*Polyribosome formation during ageing of the sliced sugar beet tissue.* Freshly cut sugar beet root tissue contained a fairly high level of ribosomes which, however, consisted almost exclusively of the monomer population (Fig. 1 and Table 1). After excision of the tissue, monoribosomes associated instantaneously into polyribosome structures, and this process was continued up to the 6th hour. At this time, polyribosome formation seemed to be completed, and the percentage distribution of mono- and polyribosomes was 34 and 66%, respectively; this remained nearly constant thereafter (Table 1). It should be noticed that this transition from free ribosomes to polysomal aggregates proceeded presumedly within preexisting ribosome pool, since only a relatively small increase in the total RNA isolated from polyribosomal complex was observed over the 0 - 18 h period (Table 1). After breaking tissue dormancy, as early as within the first hour of ageing, the newly synthesized RNA fraction entered the polyribosomal complex (Fig. 2a). This early synthesized RNA (after being released from polyribosomes) sedimented in 5 - 25%

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Table 1

*Rate of polyribosome formation over 0 - 18 h period after breaking the dormancy of sugar beet root tissue*

Percentage of polyribosomes was calculated taking as 100 total u.v. absorbing material. RNA in the ribosomal fraction is expressed as milligrams of total ribosomal RNA (i.e. mono-+poly-ribosomes) isolated from 10 g of fresh tissue.

| Time of ageing (h) | Number of determinations | Polyribosomes (%) | Total ribosomal RNA |
|---|---|---|---|
| 0 | 3 | 12 | 0.54 |
| 0.5 | 2 | 37 | 0.52 |
| 1 | 2 | 51 | 0.54 |
| 2 | 5 | 57 | 0.53 |
| 6 | 6 | 66 | 0.61 |
| 8 | 2 | 68 | 0.64 |
| 12 | 2 | 68 | 0.67 |
| 18 | 6 | 70 | 0.69 |

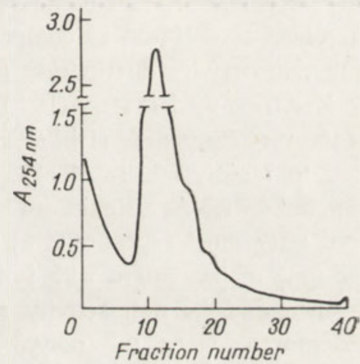


Fig. 1. Distribution pattern of ribosomes from dormant tissue of sugar beet root. Freshly cut tissue (10 g) was used. For details of the isolation procedure see Methods.

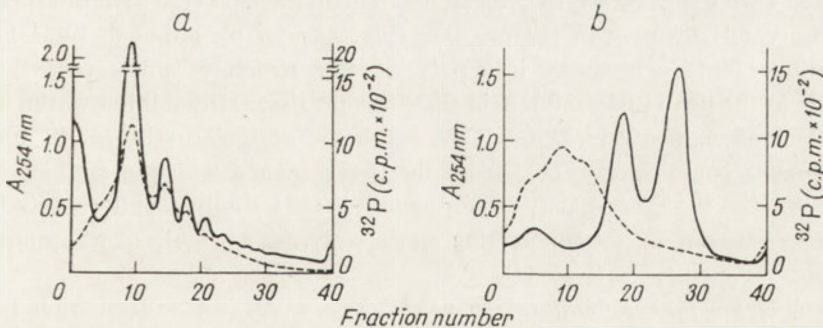


Fig. 2. Sucrose density gradient profiles of polyribosomes (*a*) and RNA released from polyribosomes (*b*). The tissue sample (15 g) was aged for 1 h in the presence of 1 mCi of [$^{32}$P]phosphate. ——, Absorbance at 254 nm; - - -, radioactivity.

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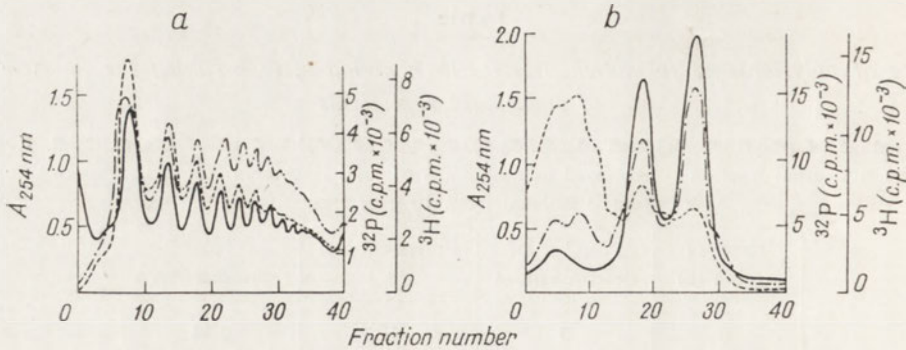


Fig. 3. Sucrose density gradient profiles of polyribosomes (*a*), and RNA released from polyribosomes (*b*). The tissue sample (15 g) was aged for 18 h under standard conditions; 100 μCi of [5-$^{3}$H]uridine was introduced 6 h, and 1 mCi of [$^{32}$P]phosphate 1 h before the end of each incubation. ——, Absorbance at 254 nm; -·-·-, [$^{3}$H]uridine radioactivity; - - -, [$^{32}$P]phosphate radioactivity.

sucrose gradient as a rather broad zone located in the upper portion of the gradient, clearly apart from 28S and 18S rRNAs (Fig. 2b). On prolonged incubation, the polyribosomal complex became progressively labelled and after 18 h its radioactivity increased several-fold as compared with the 1 h stage of tissue ageing (Fig. 3a). The radioactivity distribution pattern of the RNA released from polyribosomes derived from the late stage (18 h) of tissue ageing (Fig. 3b) differed from that obtained at the very early stage (1 h) (Fig. 2b). Under the same labelling conditions, in addition to rapidly labelled RNA, sedimenting as a heterodisperse fraction between 18S and 4S RNA, a significant labelling of lighter and heavier rRNAs was observed even after short exposure (1 h) to the radioactive RNA precursor (Fig. 3b). When the time of incubation with isotope was prolonged to 6 h, rRNA became heavily labelled and its radioactivity represented about 75% of the total radioactivity incorporated into the polyribosomal complex (Fig. 3b).

*Effect of inhibitors of RNA synthesis upon labelling and formation of polyribosomes.* Incorporation of labelled precursors into RNA of polyribosomes was totally prevented during the first 6 h of tissue ageing if actinomycin D (20 μg/ml) was present from the very beginning in the medium (Fig. 4a and b), although some limited association of monoribosomes into polyribosome structures still occurred, probably due to the preexisting mRNA in the dormant tissue. In addition to the inhibitory effect of actinomycin D on RNA synthesis, a significantly smaller amount of ribosomes could be extracted from the tissue treated with the antibiotic (Fig. 4a and b). On the other hand, 5-fluorouracil ($10^{-3}$ M) only slightly reduced [$^{3}$H] uridine incorporation, not suppressing appreciably the assembly of ribosomes into polyribosome structures (Fig. 4c).

*Effect of the RNase treatment on polyribosomes.* As can be seen from Fig. 5a and b, brief treatment with very low concentration (0.1 μg/ml) of pancreatic RNase converted heavy polyribosomes almost quantitatively to monomers and dimers, most of the radioactivity being shifted to the top of the gradient. Easy dissociation

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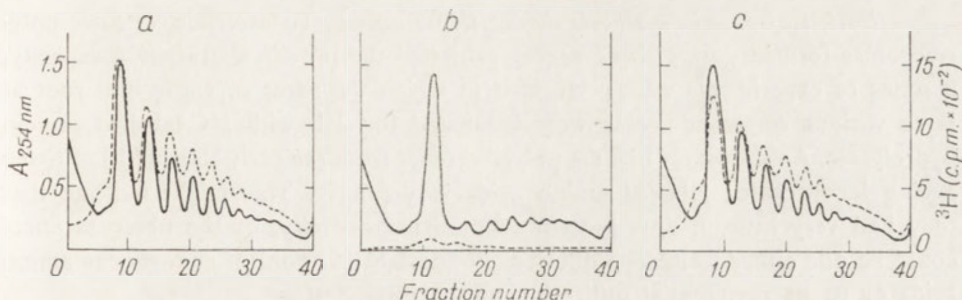


Fig. 4. Effect of actinomycin D and 5-fluorouracil treatment on polyribosome formation and [$^3$H]uridine incorporation into RNA of polyribosomes in sugar beet slices aged for 6 h. The tissue samples (7.5 g) were aged under standard conditions (*a*), in the presence of 20 μg/ml of actinomycin D (*b*), or $10^{-3}$ M-5-fluorouracil (*c*). One hour before the end of incubation 50 μCi of [5-$^3$H]uridine was introduced. Polyribosomes were extracted and analysed as described under Methods. ——, Absorbance at 254 nm; - - -, radioactivity.

of polyribosomes indicates that mRNA engaged in polyribosomal complex had been chopped. This directly proves that continuity of mRNA thread is a prerequisite for association of monoribosomes into polyribosome structures and rules out the possibility of artificial aggregation of monomers during the extraction procedure. The remaining radioactivity sedimenting with monomers and dimers probably represents only in part short fragments of mRNA still attached to ribosomes and in part the labelled rRNA which seems to be preserved under very mild conditions of RNase digestion. However, raising the RNase concentration to 2 μg/ml resulted in ribosome destruction followed by complete RNA degradation, both the radioactivity and the u.v. absorbing material being shifted to the upper portion of the centrifuge tube (Fig. 5c).

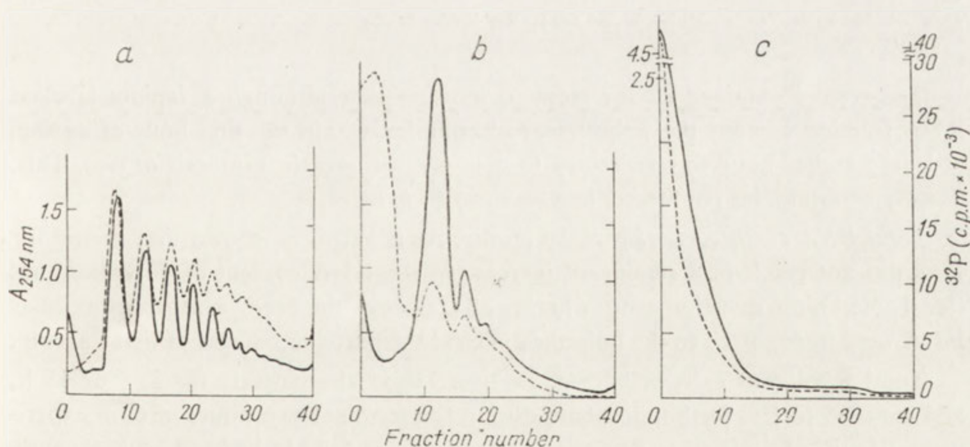


Fig. 5. Effect of RNase treatment on sedimentation and radioactivity profiles of polyribosomes. The tissue sample (10 g) was aged for 18 h; 1.2 mCi of [$^{32}$P]phosphate was introduced to the medium 1 h before the end of incubation. Control (*a*); RNase-treated at concn. of: 0.1 μg/ml (*b*), and 2 μg/ml (*c*). Polyribosomes were fractionated by sucrose density gradient centrifugation. For other details see Methods. ——, Absorbance at 254 nm; - - -, radioactivity.

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*Activation of protein synthesis during tissue ageing*. To ascertain whether polyribosomes formed during tissue ageing exhibited the protein synthesizing capacity, a series of experiments were performed in which the slices of sugar beet root at three various stages of ageing were incubated for 2 h with $^{14}C$-labelled protein hydrolysate. As shown on Fig. 6a, polyribosomes from the early stage (2 h) of tissue ageing incorporated labelled amino acids very poorly. This might indicate that they had very little, if any, protein synthesizing capacity, or the observed effect could be due either to isotope dilution by non-labelled pool of endogenous amino acids or to its preferential utilization at the earliest stage.

Polyribosomes extracted from the tissue at the later stage (6 h) were far more efficient in protein synthesis (Fig. 6b), whereas those derived from the 18 h stage showed a very high rate of amino acid incorporation, the radioactivity of the polyribosomal complex reflecting the amount of nascent protein (Fig. 6c).

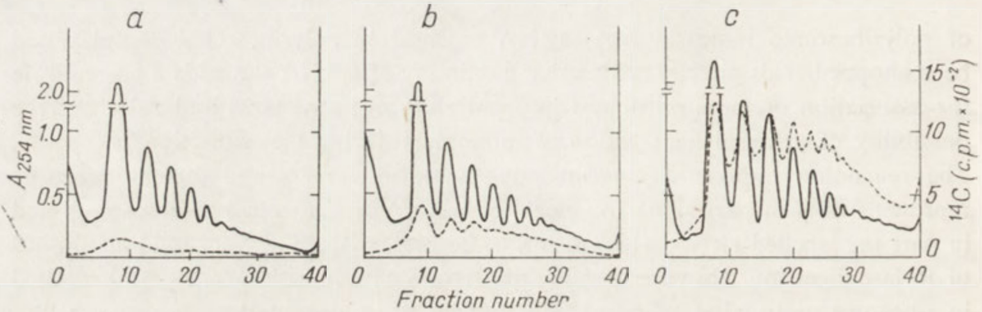


Fig. 6. Effect of tissue ageing on activation of protein synthesis *in vivo*. Three 7.5 g samples of sugar beet slices were aged for 2 h (*a*), 6 h (*b*), and 18 h (*c*); 100 μCi of $^{14}C$-reconstituted protein hydrolysate was added 2 h before the end of incubation. For details see Methods. ——, Absorbance at 254 nm; - - -, radioactivity.

The results obtained so far seem to indicate that although a rapidly labelled RNA fraction entered the polyribosomal complex within the first hour of ageing, at least another hour was necessary to demonstrate protein synthesis *in vivo*. This, however, should be confirmed in experiments *in vitro*.

*Total RNA synthesis during tissue ageing*. As it had been shown that ageing for 18 h did not result in a significant increase in the RNA content of polyribosomes (see Table 1), the question arose whether all classes of the newly synthesized cellular RNA were transported to the polyribosomes at the particular stages of tissue ageing.

Total RNA was extracted from the tissue aged alternatively for 2, 6 or 18 h, and exposed to [$^{32}P$]orthophosphate during the terminal two hours of each experiment. The RNA isolated was purified as described under Methods and subsequently submitted to 5 - 25% sucrose gradient fractionation. Very characteristic differences in the labelling pattern of the total RNA were observed (Fig. 7a, b and c) on exposing the tissue from different stages of ageing to $^{32}P$ for the same time period. The analysis revealed, as it was observed for RNA released from polyribosomes isolated at 1 h

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of ageing (Fig. 2b), that at the early stage (2h) most of the radioactive newly synthesized RNA sedimented in the upper portion of the tube with a very heterodisperse pattern (Fig. 7a). At this stage, relatively low labelling of both rRNAs was observed.

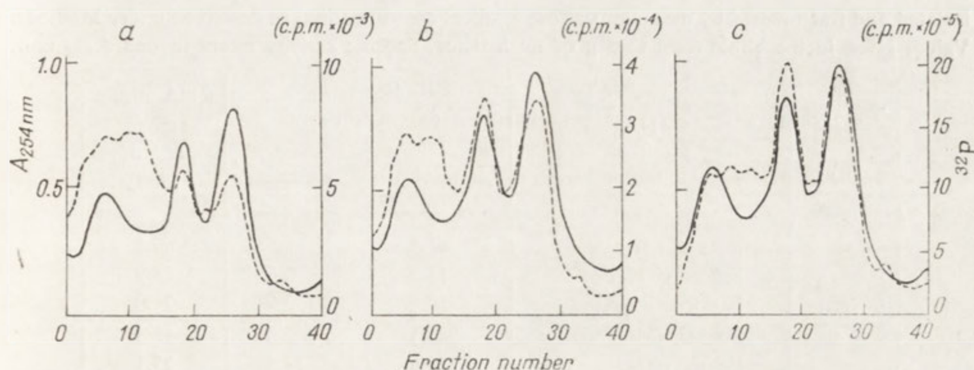


Fig. 7. Sucrose gradient sedimentation and radioactivity profiles of total RNA at three stages of tissue ageing. Three 15 g samples of sugar beet slices were aged for 2 h (*a*), 6 h (*b*), and 18 h (*c*); 1.2 mCi of [$^{32}$P]phosphate was introduced 2 h before the end of incubation. Total RNA was isolated and analysed as described under Methods. ——, Absorbance at 254 nm; - - -, radioactivity.

On further ageing (Figs. 7b and c), a dramatic acceleration of the rate of rRNA synthesis took place. At the same time, the respective increase in the rate of labelling of the "mRNA+tRNA" fraction became smaller, as compared with the rRNA fraction.

A net increase — up to 43% of the initial value — was also observed in the total RNA content per gram of fresh weight throughout the 0 - 18 h period.

Calculation of specific activity of lighter and heavier rRNAs showed that the former was at all stages of tissue ageing labelled to a higher extent than the heavier rRNA (Table 2). This disparity was especially pronounced at the early stage of tissue ageing (2 h) when the specific activity of lighter rRNA was nearly twice as high as that of heavier rRNA; and at the late stage (18 h) it still remained by about 30% higher.

The contribution of particular RNA fractions to the total RNA radioactivity at the three stages of ageing is given in Table 2. At the early stage the radioactivity of "mRNA+tRNA" fraction comprised roughly two-thirds of the total RNA radioactivity, but with prolonged ageing its contribution dropped to about one-third, whereas the radioactivity of both rRNAs increased up to one-third each.

This observation indicates that the newly synthesized rRNA accumulated relatively faster than the mRNA+tRNA fraction. This might be conceivably explained in terms of considerably longer half-life of rRNA.

To make a direct estimate whether all the newly synthesized RNA entered polyribosomal complex, an additional experiment was performed in which three 15 g batches of sugar beet root slices were aged for 2, 6 or 18 h and labelled with [$^{32}$P] phosphate for the final two hours. After the incubation was terminated, each sample

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Table 2

*Synthesis of particular RNA classes at three stages of sugar beet ageing*

Three 15 g batches of tissue slices were aged in 10 ml of the medium for the time indicated. Two hours before the end of incubation 1.2 mCi of [$^{32}$P]phosphate was introduced. Total RNA was isolated and fractionated by means of sucrose gradient centrifugation, as described under Methods. Values given in the Table refer to 5 g of fresh tissue. Specific activity refers to one $A_{260}$ unit.

| Time of ageing (h) | RNA fraction | Radioactivity (c.p.m. × $10^{-3}$) | | RNA radio-activity (% of total) |
|---|---|---|---|---|
| | | total | specific | |
| 2 | mRNA+tRNA | 42.3 | 38.5 | 60.1 |
| | lighter rRNA | 13.2 | 6.6 | 18.9 |
| | heavier rRNA | 14.6 | 3.9 | 21.0 |
| 6 | mRNA+tRNA | 242.3 | 142.8 | 38.5 |
| | lighter rRNA | 170.5 | 58.6 | 27.1 |
| | heavier rRNA | 216.7 | 42.3 | 34.4 |
| 18 | mRNA+tRNA | 8048.6 | 3908.4 | 30.9 |
| | lighter rRNA | 8859.3 | 2682.8 | 34.1 |
| | heavier tRNA | 9120.1 | 1827.9 | 35.0 |

was divided into two equal parts from which polyribosomal RNA and total RNA were extracted in parallel.

Figure 8 illustrates the ratio of the total amount of the newly synthesized cellular RNA to its portion recovered from the polyribosomes at the three stages of tissue ageing. At 2 h after breaking tissue dormancy, nearly 80% of the newly synthesized RNA was found in the polyribosomal fraction, whereas at the late stage (18 h) only 62% of the total radioactive RNA entered polyribosomes. One possible explanation of this result is that the specific population of RNA which was transcribed at the later stage and did not leave the nucleus, could account for this difference. To check this assumption, nuclear RNA was analysed.

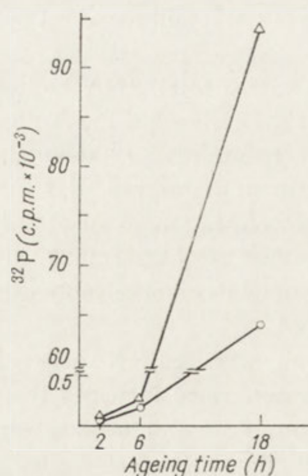


Fig. 8. Rate of [$^{32}$P]phosphate incorporation into polyribosomes (○) and total RNA (△). For experimental conditions see legend to Fig. 7. For other details see Methods.

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*Nuclear RNA labelling at the early and late stages of tissue ageing.* The nuclei were isolated from the sugar beet root slices aged for 2 or 18 h, and purified as described under Methods. The total nuclear RNA was extracted therefrom using the hot phenol technique. Data given in Table 3 confirm the expected increase in the rate of RNA synthesis between the 2 and 18 h of tissue ageing. To see whether this acceleration of RNA synthesis was accompanied by any qualitative changes

Table 3

*Specific activity of nuclear RNA at early and late stage of tissue ageing*

For experimental conditions of tissue ageing and labelling see legend to Table 2. Other details given in Methods.

| Time of ageing (h) | Specific radioactivity (c.p.m. $\times 10^{-4}/A_{260}$ unit) |
|---|---|
| 2 | 13 |
| 18 | 1852 |

in the newly synthesized RNA population, nuclear RNA preparations derived from the tissue aged for 2 or 18 h, were submitted to the sucrose gradient ultracentrifugation (Fig. 9a, b). The comparison of radioactivity profiles of nuclear RNA synthesized at those two stages clearly indicated not only quantitative, but also some qualitative changes in the pattern of RNA transcription. The most obvious difference consisted in a considerable contribution of 28S and 18S rRNAs and their precursor to the radioactivity of nuclear RNA at the late stage (Fig. 9b), whereas at the early stage most of the newly synthesized RNA was represented by a polydisperse RNA population sedimenting slower than 18S rRNA (Fig. 9a).

It is also noteworthy that the highest radioactivity of nuclear RNA at the 18 h stage of ageing, was found in the fraction sedimenting with 18S rRNA, i.e. RNA which is known to be a very early product of the ribosomal precursor cleavage

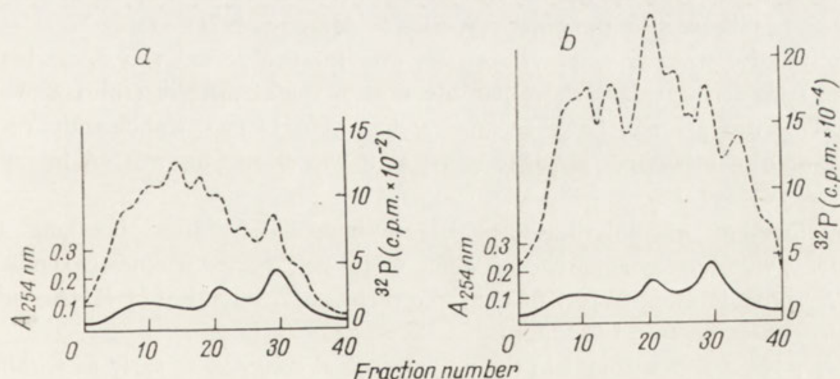


Fig. 9. [$^{32}$P]Phosphate incorporation into nuclear RNA after 2 h (*a*) and 18 h (*b*) of ageing of tissue slices. For experimental conditions see legend to Fig. 7. Total nuclear RNA was isolated and analysed as described under Methods. ——, Absorbance at 254 nm; - - -, radioactivity.

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(Penman, 1966; Seitz & Seitz, 1972). This is in agreement with our observations (Fig. 7 and Table 2) that the lighter rRNA showed higher specific activity as compared with its heavier counterpart.

## DISCUSSION

The rapid association of monoribosomes into polyribosome structures is one of the earliest metabolic events triggered by breaking the dormancy of sugar beet root. Polyribosomes hardly detectable (12%) in the freshly cut tissue begin to develop almost instantaneously after the sliced tissue has been exposed to the optimal temperature (30°C). The rate of polyribosome formation, very high during the first two hours, decelerates gradually and the maximum level of polyribosomes (66%) reached at the 6th hour of tissue ageing remains nearly constant on ageing prolonged for the next 12 h. Our results are essentially consistent with the data obtained for carrot roots (Leaver & Key, 1967) and potato tubers (Kahl, 1971). Polyribosomes isolated by us from the aged slices of sugar beet roots were much less degraded (in our preparations dodecamers were present, whereas in the preparations from the carrot and potato, at most hexa- and heptamers could be detected). Our data, in contrast to the results of Leaver & Key (1967) and Kahl (1971) clearly indicate that under conditions which prevented transcription completely, some limited polyribosome formation still occurred (Fig. 1 and Fig. 4b).

This suggests that the synthesis of a new mRNA during the initial phase might not be strictly required and most likely messenger molecules already preexisting in dormant tissue could be utilized for the earliest polyribosome formation. Such a preformed mRNA might play a similar role in plant storage organs as does the so-called "maternal" mRNA which was found to be stored awaiting activation in the unfertilized sea urchin eggs (Gross *et al.*, 1964; Spirin & Nemer, 1965; Gross, 1967; Tyler, 1967). This stored message is released for translation in the early post-fertilization stages (Infante & Nemer, 1967; Rinaldi & Monroy, 1969), and is responsible for at least 85% of protein synthesizing capacity (Humphreys, 1971). More recently an evidence for the existence of preformed mRNA pool in the wheat embryos has been also presented (Weeks & Marcus, 1971).

The relative stability of polyribosomes over the 6 h period was somewhat surprising, considering rather short half-life time of plant mRNA (Johri & Varner, 1970). One can assume, however, that actinomycin D may significantly prolong the life-time of messenger in sugar beets, as it was shown for mRNA in rat liver (Endo *et al.*, 1971).

Development of polyribosomes began immediately after breaking tissue dormancy, without any apparent lag which might be expected if a newly synthesized mRNA were a limiting factor. At the same early stage, initiation of RNA synthesis was also observed (see Fig. 2a, b).

This RNA fraction found in the polyribosomal complex as early as within one hour after breaking tissue dormancy (Fig. 2b) shows a characteristic heterodisperse sedimentation pattern, typical for messenger fraction (Noll & Stutz, 1968). At this stage no labelling of 28S and 18S rRNAs was detected. This might suggest that the

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renewal of genomic activity begins with preferential transcription of mRNA, while genes coding for rRNA become "switched on" apparently at the later stage.

Appreciable [$^3$H]adenosine incorporation into polyribosomes of carrot root washed for 1 h has been also demonstrated (Leaver & Key, 1967), but no attempt was made to distinguish which class of RNA was labelled at this early stage. On the other hand, potato tuber slices aged for 1 h appeared to be still inactive in resumption of RNA synthesis (Kahl, 1971).

Although newly synthesized rRNA could be detected as early as at the 2 h stage of ageing (see Fig. 7a), the development of polyribosomes does not seem to rely exclusively on the supply of new ribosomes. Since 5-fluorouracil, a specific inhibitor of rRNA and sRNA synthesis in plant systems (Key & Ingle, 1968) had very little effect on formation of polyribosomes and their labelling, we might conclude contrary to Kahl (1973) that the synthesis of neither rRNA nor tRNA was essential for polyribosome development during the 6 h period of tissue ageing. Instead, an apparent need for continuous supply of mRNA was shown (Fig. 4a and b) and it may be postulated that both the preexisting and the newly synthesized messengers are essential for the early polyribosome formation in sugar beet root, as it was observed in the early embryonic development of sea urchin (Kedes & Gross, 1969; Humphreys, 1971).

Data on the pattern of total cellular RNA labelling during tissue ageing confirm the results obtained with the polyribosomal fraction: at the early stage (2 h) a polydisperse mRNA-like RNA fraction was predominantly synthesized (Fig. 7a). Proportion of the radioactivity which may be ascribed to this RNA species to the radioactivity of the total RNA shows, however, a decreasing tendency on ageing of the tissue, whereas at the same time a rapid accumulation of 28S and 18S rRNAs was noted (Table 2, Fig. 7a, b, c). This is probably a function of the acceleration of RNA synthesis with tissue ageing and distinctly different life-time of mRNA and rRNA. Half-life time of the presumed mRNA fraction in plants varies from 10 to 15 min for peas (Loening, 1965; Johri & Varner, 1970) and does not exceed 2 h for peanut cotyledons (Chroboczek & Cherry, 1966) and soybean hypocotyl (Key & Ingle, 1964). Therefore, high accumulation of the radioactive label in the presumed mRNA fraction during 2 h exposure to [$^{32}$P]phosphate should not be expected. On the other hand, in the fast growing cultures of *Lemna minor* half-life time of rRNA has been estimated to be between 5 and 8 days (Trewavas, 1970). Thus, it is clear that any rRNA synthesized in the initial phase of incubation with isotope, will be recovered during extraction, whereas due to the high turnover rate, only a part of the labelled messenger fraction may be still present in the tissue at the end of incubation.

This assumption is in agreement with the pattern of the label distribution in RNA released from polyribosomal fraction of the tissue aged for 18 h following incubation with [$^3$H]uridine for 6 h and with [$^{32}$P]phosphate for 1 h (Fig. 3b). It may be concluded that prolonged time of labelling apparently favours the synthesis of more stable classes of sugar beet RNA.

The significant difference in labelling of lighter and heavier rRNAs (Fig. 7a, b and Table 2) can easily be explained by shorter time of 18S rRNA maturation

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within the nucleolus. It has been demonstrated for mammalian tissues (Penman, 1966; Weinberg & Penman, 1970) as well as for plants (Seitz & Seitz, 1972; Cecchini *et al.*, 1972) that 45S rRNA precursor is quickly split into 2 components: an 18S rRNA, which leaves the nucleus rapidly and within 30 - 60 min is found in the cytoplasm, and the remaining part of ribosomal precursor which undergoes further processing in the nucleolus, and appears much later in the ribosomes.

Finally, the disparity observed between labelling of polyribosomal RNA and total RNA (see Fig. 8) increasing with the time of tissue ageing, may be caused by at least two reasons.

One can presume that some of the newly synthesized RNA molecules, though present in cytoplasm, were inactive in protein synthesis and thus were not recovered from tissue homogenate in the form of polyribosomal complex. In fact, a significant portion of non-ribosomal, rapidly labelled RNA may not be associated with ribosomes in cytoplasm (Georgiev, 1972) and it may correspond to the so-called free informosomes (Spirin *et al.*, 1964). These cytoplasmic ribonucleoprotein particles may contain mRNA, but are not combined with ribosomes (Samarina *et al.*, 1973).

The second possibility is that nuclear RNA population which does not serve as precursor for cytoplasmic RNA, and synthesis of which increases with the time of ageing, may be responsible for the disparity in labelling of total cellular RNA and RNA of the polyribosomal fraction. It was shown first by Harris (1963) and Scherrer *et al.* (1963) and fully confirmed by others (Roberts, 1965; Houssais & Attardi, 1966; Georgiev, 1972) that only part of RNA synthesized in the cell nucleus is transported to the cytoplasm. Georgiev (1972) estimates that about one-half to three-fourths of the newly synthesized RNA molecules are processed within the nucleus, never reaching cytoplasm. On the other hand, it was shown in the experiments on saturation of DNA with the increasing amounts of RNA that the nuclear RNA saturates about 5 - 10 times more DNA than does cytoplasmic mRNA (Shearer & McCarthy, 1967; Scherrer *et al.*, 1970). This means that the newly synthesized population of heterogeneous nuclear RNA represents two general classes of sequences: functional sequences, which survive processing of the giant pre-mRNA and correspond to the portion of molecule which is transported to the cytoplasm, and "service" sequences, which are degraded within the cell nucleus. The significance of intranuclear RNA turnover has not yet been elucidated, although a plausible suggestion has been made that it may be engaged in the regulation of genome activity (Pontecorvo, 1966; Georgiev, 1972). Therefore, it appears quite likely that due to the increasing genome availability during ageing of the sugar beet tissue slices (Duda & Cherry, 1971) the population of RNA strictly confined to the nucleus was increased (Fig. 8) and this may account for the difference observed in our experiments between the radioactivity of polyribosomal and total RNA.

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## POWSTAWANIE POLIRYBOSOMÓW I SYNTEZA RNA PO PRZERWANIU SPOCZYNKU KORZENIA BURAKA CUKROWEGO

### Streszczenie

1. Przerwanie stanu spoczynku korzenia buraka cukrowego wywołane pokrojeniem tkanki powoduje natychmiastową asocjację obecnych w tkance rybosomów w polirybosomy; maksymalny poziom (66%) polirybosomów obserwowany po 6 godz. inkubacji utrzymuje się przez następnych 12 godz.

2. Jakkolwiek synteza RNA rozpoczyna się już w pierwszej godzinie, to jednak wczesne powstawanie polirybosomów nie wydaje się być ściśle uzależnione od syntezy RNA, gdyż proces ten odbywa się także pomimo całkowitego jej zahamowania. Sugeruje się, że istniejący w spoczynkowej tkance mRNA jest wykorzystywany w tworzeniu polirybosomów we wczesnym stadium rozwoju tkanki.

3. Przywrócenie syntezy RNA po przerwaniu spoczynku tkanki rozpoczyna się preferencyjną syntezą frakcji mRNA, podczas gdy inicjacja syntezy rRNA następuje w późniejszym okresie.

4. Procesowi uporządkowanej derepresji genomu towarzyszy zwiększona synteza pewnej frakcji RNA, która nie jest wykorzystywana w tworzeniu polirybosomów i która być może reprezentuje populację RNA, którego metabolizm jest ściśle ograniczony do terenu jądra.

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# HYBRIDIZATION OF LABELLED RNA TO DNA FROM SUGAR BEET ROOT

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1. The RNA population undergoes qualitative changes during the first 18 h after breaking the dormancy of sugar beet root. 2. The results of competitive RNA - DNA hybridization experiments indicate that population of "late" (18 h) RNA contains about 40% of "early" (6 h) RNA transcripts. It is suggested that the change in the degree of RNA heterogeneity is due to the synthesis of different mRNA and/or specific nuclear RNAs.

Hybridization of RNA to homologous DNA has become a useful tool for examination of redundant DNA sequences in bacteria (Dubnau *et al.*, 1965; Kennell, 1968) and eukaryotic organisms (Britten & Kohne, 1968; Church & McCarthy, 1968; Paul, 1970; Walker, 1971) and determining qualitative changes in unique DNA sequences of different tissues and during various stages of cellular differentiation (Gelderman *et al.*, 1968; Davidson & Hough, 1969; Church & McCarthy, 1970; Gelderman *et al.*, 1971; Laird, 1971; Brown & Church, 1971). The differentiation of cells is regulated by the transcription of specific unique DNA sites, which in turn leads to the synthesis of specific proteins.

We have employed the RNA - DNA hybridization method for studying the control of differentiation processes in sugar beet root. When this tissue is sliced into thin sections and aerated in buffer, a considerable increase in metabolic activity including a sizable increase in the rate of RNA and protein synthesis is noted (Wasilewska & Cherry, 1974). These changes in metabolic activity may be due either to the primary changes in the template activity of DNA or in the activity of RNA polymerases.

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The aim of the present work was to determine whether these changes in metabolic activity resulted in the appearance of different mRNAs during various stages of ageing of sugar beet root tissue.

## MATERIALS AND METHODS

*Chemicals.* The following chemicals were used: [5-$^3$H]uridine, spec. act. 8 Ci/mmol (Schwarz Mann Bioresearch, Orangeburg, N.Y., U.S.A.); [$^{32}$P]orthophosphoric acid in 0.01 M-HCl or aqueous solution, carrier-free (New England Nuclear Corporation, Boston, Mass., U.S.A.); DEAE-cellulose Whatman DE-23 (W. and R. Balston Ltd., Maidstone, England); DNA from calf thymus, type 1, sodium salt (Sigma Chemical Company, St. Louis, Mo., U.S.A.); DNase, RNase-free (Worthington Biochemical Corporation, Freehold, N.Y., U.S.A.); hydroxyapatite, Bio-Gel-HTP (BioRad. Lab., Richmond, Calif., U.S.A.) and pronase (Calbiochem, Los Angeles, Calif., U.S.A.).

*Plant material.* Sugar beets (*Beta vulgaris* L) were grown in the field and stored after harvest at 4°C. Fresh and aged tissue was used for obtaining DNA and RNA preparations. Experimental conditions for ageing of the tissue and labelling were the same as in the accompanying paper (Wasilewska & Cherry, 1974). Green pea seedlings (*Pisum sativum* L var. Burpeeana) were grown in Vermiculite under standard conditions for 14 days.

*DNA isolation and purification.* Unlabelled DNA was isolated from plant tissue by the standard SDS-EDTA-SSC[1] technique according to Marmur (1961), followed by treatment with pronase and RNase. The DNA preparation was subjected to further purification by fractionation on hydroxyapatite (Britten *et al.*, 1970). DNA eluted from the column with 0.4 M-phosphate, pH 7.0, was dialysed overnight against $0.01 \times$ SSC - $10^{-4}$ M-$MgCl_2$, and stored at 2°C until use. This procedure yielded 11.5 mg of double-stranded DNA from 2 kg of sugar beet tissue and 6.3 mg from 500 g of pea seedlings. DNA was determined by the diphenylamine method of Burton (1956), and the purity was examined by measuring u.v. absorption between 210 and 310 nm.

*Loading filters with denatured DNA.* Concentration of NaCl in the DNA solution was adjusted to 0.015 M and the DNA was denatured by heating for 10 min at 95°C and rapidly cooled. It was shown, using the hyperchromicity test as an index of denaturation, that the obtained DNA preparations from sugar beet and peas gave 33 and 25% increase in absorbancy at 260 nm over the respective native DNA solutions. The salt concentration of the denatured DNA solution was adjusted to $4 \times$ SSC and DNA concentration to 1 - 5 μg/ml.

Nitrocellulose filters (type B-6, 25 mm from Schleicher & Schuell) were presoaked in $4 \times$ SSC and washed with 20 ml of $4 \times$ SSC. Wet filters were slowly loaded (1 ml/min) with the desired amount of denatured DNA in 5 or 10 ml of $4 \times$ SSC. Then both sides of the loaded filters were rinsed twice with 50 ml of $4 \times$ SSC. During DNA loading, the first 10 ml portion of the filtrate was collected to determine the amount

[1] SSC is standard saline citrate, 0.15 M-NaCl - 0.015 M-trisodium citrate.

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of DNA retained on the filter. The DNA loaded filters were dried at room temperature for a least 4 h and then at 80°C for 2 h. Then they were stored in a sealed box at 4°C for several weeks without any loss of DNA. Blank filters without DNA were subjected to the same procedure.

*Determination of DNA retention on filters.* From each set of filters loaded with DNA, 8 - 10 were randomly selected to determine DNA retention. Filters were placed in vials and 1 ml of DNase solution (10 μg/ml) containing 0.1 × SSC and $10^{-2}$ M-$MgCl_2$ was added. Samples were incubated for 12 h at 30°C with gentle shaking. The concentration of deoxyribonucleotides was determined on portions of the digest by the diphenylamine method (Burton, 1956). Routinely 70 - 82% of plant DNAs loaded onto filters was retained while in the case of calf thymus DNA nearly 100% retention was achieved under the same conditions.

*RNA isolation and purification.* Total RNA was isolated from the aged sugar beet tissue labelled with radioactive precursors, by the standard phenol-SDS extraction technique as previously described (Wasilewska & Cherry, 1974). DNA was removed by digestion with DNase (20 μg/ml in 0.1 × SSC and $10^{-2}$ M-$MgCl_2$ for 20 min at 4°C) which subsequently was extracted with SDS-phenol. RNA was then precipitated with 2 vol. of 95% ethanol containing 0.2 M-potassium acetate, the precipitate was dissolved in 0.01 × SSC and dialysed against the same solution. Concentration of NaCl in the DNA solution was adjusted to 0.6 M (equal to 4 × SSC) and finally the RNA solution was diluted to 10 $A_{260}$ units/ml.

Nuclear RNA was obtained from the nuclei isolated according to Rho & Chipchase (1962) as previously described (Wasilewska & Cherry, 1974).

RNA released from polyribosomes was obtained as previously described (Wasilewska & Cherry, 1974). RNA reprecipitated with ethanol was dissolved in cold 0.01 M-Tris-HCl buffer, pH 7.2, containing 0.01 M-$MgCl_2$ and was subjected to DNase digestion (20 μg/ml) for 20 min at 4°C, followed by removal of the enzyme by SDS-phenol extraction. RNA was recovered from the aqueous phase by precipitation with 3 vol. of cold (-20°C) ethanol. The purified RNA preparations (nuclear, total, or released from polyribosomes) were submitted to sucrose density gradient centrifugation as previously described (Wasilewska & Cherry, 1974).

Gradients were analysed at 254 nm on an ISCO sucrose density gradient fractionator; from each tube 40 fractions, 0.6 ml each, were obtained, and their radioactivity was monitored. Three fractions of RNA were collected as based on u.v. absorbtion at 254 nm: a) mRNA and tRNA, b) lighter rRNA, and c) heavier rRNA. The respective fractions from several tubes were combined, and the absorption at 260 nm and radioactivity were measured.

*Purification and concentration of RNA fractions.* The mRNA + tRNA fraction was concentrated by DEAE-cellulose chromatography. Three small columns (0.8 cm × 15 cm) were packed with 2 ml of DEAE-cellulose suspended in 0.01 × SSC and the RNA fraction was absorbed at a low flow rate of 10 - 15 drops/min. The columns were rinsed with about 200 ml of 0.01 × SSC to remove the sucrose gradient components. RNA was eluted with a small volume of 4 or 6 × SSC and its content was determined by measuring absorption at 260 nm. The recovery was 70 - 78%.

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The DEAE-cellulose chromatography appeared to be useful only for the mRNA+tRNA fraction. Since under these conditions the recovery of both rRNA fractions was very poor (15 - 25%) they were recovered from the sucrose gradient by precipitation with ethanol and dissolved in 4×SSC.

*Standard hybridization procedure.* Hybridization of labelled RNA to homologous DNA was carried out in a two-phase system with RNA dissolved in 4×SSC solution and DNA immobilized on nitrocellulose membrane filters, according to Gillespie & Spiegelman (1965). Filters were loaded with DNA and cut into 9 sections (mini-filters). The DNA filters were then placed in small vials fitted with plastic caps and the RNA samples (in 0.5 ml or 1 ml of 4×SSC) were added. The RNA was allowed to anneal to DNA for 18 h at 65°C in a water bath with moderate shaking, followed by 2 h standing in ice. Sections of filters were removed and rinsed with 200 ml of 2×SSC on a Büchner funnel covered with Miracloth, then the filters were placed on a Millipore funnel and washed with 100 ml of 2×SSC using moderate suction. Filters were transferred to vials containing 1 ml of the RNase solution (20 μg/ml of pancreatic RNase, DNase-free; heated before use at pH 5.0 for 10 min at 80°C). Filters after annealing were incubated for 2 h at room temperature to remove the non-specifically bound RNA. After RNase digestion the filters were washed thoroughly with 200 ml of 2×SSC using a Millipore funnel, dried and the radioactivity remaining on the filters was counted in a Packard Tri-Carb scintillation spectrometer.

"Noise" (non-specific binding) was monitored by incubating blank filters (without DNA) in a labelled RNA solution, followed by the same treatment as applied to DNA-loaded filters. The amount of radioactivity remaining on the blank filters was subtracted from that of the hybridizable radioactivity.

## RESULTS

*Hybridization of RNA from three stages of tissue ageing to homologous DNA.* Changes in hybridizable RNA species extracted during ageing of sugar beet tissue were examined. Slices of sugar beet roots were aged for 2, 6 and 18 h and labelled with [$^{32}$P]phosphate during the final 2 h of each incubation. Increasing amounts of total RNA extracted from each sample were annealed to a constant amount of filter-bound DNA (see Methods). Saturation curves (Fig. 1) indicate that RNA samples from the tissue aged for a few hours (2 and 6 h) saturate DNA at RNA:DNA ratio of 1 (Fig. 1a and b) whereas twice as much of RNA isolated from the tissue aged for 18 h is required for saturation of a given amount of DNA (Fig. 1c). We assume therefore that new species of RNA start to appear between the 6th and 18th hour of tissue ageing and become available for hybridization. The same saturation characteristics found for the two early transcribed cellular RNA populations (2 and 6 h) does not, of course, exclude the possibility that they may also differ qualitatively in composition of RNA species.

Although this set of hybridization experiments was performed with total RNA which represents three major classes of cellular RNA, it seems most likely that the

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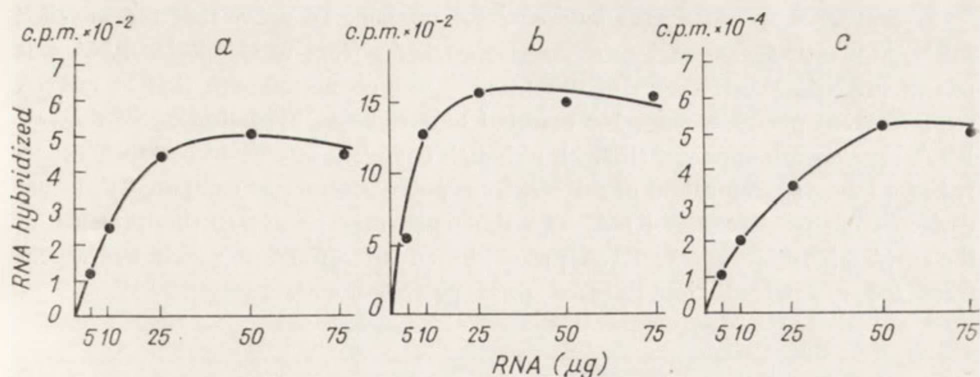


Fig. 1. Hybridization of $^{32}$P-labelled RNA from sugar beet root to homologous DNA. RNA isolated from tissue aged for: *a*, 2 h (spec. act. 375 c.p.m./μg); *b*, 6 h (spec. act. 1480 c.p.m./μg); *c*, 18 h (spec. act. 48 917 c.p.m./μg) was annealed to 25 μg filter-bound sugar beet DNA in 0.5 ml of 4×SSC for 18 h at 65°C.

rapidly labelled RNA (possibly mRNA and its nuclear precursor) accounts for this difference. Since it is well known that rRNA and tRNA are transcribed from a very small, less than 0.5%, portion of the genome (Ritossa & Spiegelman, 1965; Chen & Osborne, 1970) we assume that complementary sites on DNA for rRNA and tRNA were rapidly saturated at the levels of RNA used in these experiments. Therefore, the increased amount of RNA from the 18-h stage required to saturate DNA might indicate an increase in the relative amount of mRNA and/or rapidly labelled nuclear RNA.

*Hybridization efficiency of various RNA fractions to homologous DNA*. To determine the relative degree of hybridization of the various classes of RNA, the total RNA was fractionated by sucrose density gradient centrifugation into lighter and heavier rRNA components, and a fraction which contained both mRNA and tRNA. The particular RNA species were precipitated with ethanol (rRNA) or purified by DEAE-cellulose chromatography (mRNA+tRNA) (see Methods). The percentage of hybridizable RNA in each purified fraction is given in Table 1. The results show that the degree of homology between DNA and the mRNA-containing fraction is about three times higher than that for the two rRNA fractions.

Table 1

*Hybridization of particular RNA fractions from sugar beet to homologous DNA*

Fifty μg of [$^{32}$P]RNA derived from the 18 h aged tissue was annealed to 25 μg of DNA under standard conditions.

| Fraction | Radioactivity | | RNA hybridized (%) |
|---|---|---|---|
| | given (c.p.m. × $10^{-6}$) | hybridized (c.p.m. × $10^{-3}$) | |
| mRNA+tRNA | 5.36 | 61.5 | 1.2 |
| lighter rRNA | 2.22 | 8.8 | 0.5 |
| heavier rRNA | 1.79 | 5.2 | 0.3 |

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*Hybridization of the rapidly labelled RNA fraction.* To prove that the so-called mRNA fraction separated by the sucrose gradients from total cellular RNA was in fact mRNA, hybridization of this fraction was compared with that of mRNA released from polyribosomes. No essential differences in hybridization of the two RNA samples are apparent (Fig. 2), although complete saturation of DNA occurred at a lower concentration of mRNA from polyribosomes as compared with that derived from total cellular RNA. This difference may be due to the presence of the rapidly labelled nuclear RNA present in the latter fraction, which was absent from the mRNA fraction released from polyribosomes.

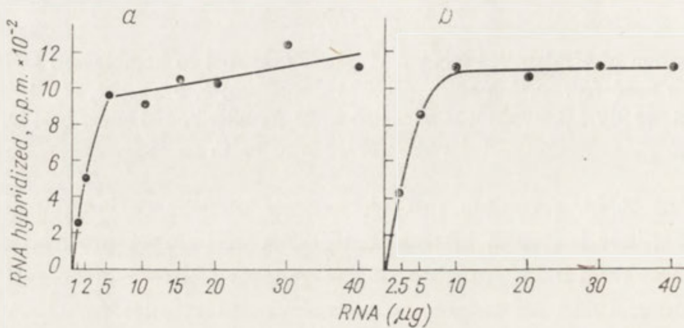


Fig. 2. Hybridization of the rapidly labelled RNA fraction to homologous DNA. This RNA fraction was isolated from total RNA (*a*) or from polyribosomes (*b*), extracted from sugar beet tissue aged for 18 h and labelled during the final 1 or 2 h with [$^{32}$P]phosphate or [$^{3}$H]uridine, respectively. The specific activities of [$^{32}$P]RNA and [$^{3}$H]RNA were 1828 c.p.m./µg and 2026 c.p.m./µg, respectively. RNA was annealed to 18 µg filter-bound sugar beet DNA in 1 ml of 4 × SSC for 18 h at 65°C.

*Hybridization of nuclear and cytoplasmic RNA fractions.* Several investigators have demonstrated the presence of heterogeneous nuclear RNA population which is restricted to the nucleus and is not transported to the cytoplasm (Attardi *et al.*, 1966; Warner *et al.*, 1966; Shearer & McCarthy, 1967). Nuclear RNA, then, represents a more polydisperse fraction than does cytoplasmic RNA. Therefore, hybridization of nuclear RNA should show more homology with DNA than cytoplasmic RNA. To test this assumption labelled RNA was extracted from sugar

Table 2

*Hybridization of nuclear and cytoplasmic RNA from sugar beet to homologous DNA*

Fifty µg of [$^{32}$P]RNA derived from the 18-h aged tissue was annealed to 50 µg of DNA under standard conditions. Cytoplasmic fraction was contaminated with nuclear fraction.

| Fraction | Radioactivity | | RNA hybridized (%) |
|---|---|---|---|
| | given (c.p.m. × $10^{-3}$) | hybridized (c.p.m. × $10^{-3}$) | |
| Nuclear | 110 | 5.9 | 5.4 |
| Cytoplasmic | 43 | 1.4 | 3.2 |

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beet nuclei according to Rho & Chipchase (1962), annealed to DNA and compared by hybridization to the cytoplasmic mRNA fraction. The results show twice as much nuclear RNA hybridization to DNA in comparison to the cytoplasmic mRNA (Table 2).

*Specificity of hybrid formation.* To check the specificity of hybrid formation, total RNA from sugar beet tissue aged for 18 h was annealed to heterologous DNAs. The data show that sugar beet RNA hybridizes very poorly with calf thymus DNA (Table 3). This result could have been anticipated since the two organisms would be expected to have very few common genes. However, sugar beet mRNA hybridized remarkably well to pea DNA in comparison with the homologous system.

Table 3

*Hybridization of mRNA from sugar beet to DNA from different sources*

Twenty five μg of [$^3$H]RNA derived from the 18-h aged tissue (spec. act. 2020 c.p.m./μg) was annealed to DNA from different sources under standard conditions.

| Source of DNA | DNA per filter (μg) | Radioactivity | | RNA hybridized (%) |
|---|---|---|---|---|
| | | given (c.p.m × $10^{-3}$) | hybridized (c.p.m.) | |
| Sugar beet | 18 | 50.5 | 1251 | 2.5 |
| Green pea | 14 | 50.5 | 425 | 0.9 |
| Calf thymus | 24 | 50.5 | 98 | 0.2 |

*Homology of RNA populations derived from the tissue at different stages of ageing.* Methods currently available to determine differences in base sequence of various RNA populations usually involve competitive hybridization. The rationale for this method is based on the assumption that two RNA molecules of common origin and similar base sequence would compete with each other for the same site on DNA, while molecules with different base sequences would not (Kennell, 1971). We attempted to determine the degree of similarity of RNA populations derived from fresh tissue slices and those aged for 6 or 18 h. The respective RNAs were extracted and compared by competitive hybridization using $^{32}$P-labelled RNA isolated from the tissue aged for 18 h. Increasing amounts of unlabelled RNAs were annealed to 25 μg of filter-bound DNA in the presence of 50 μg of $^{32}$P-labelled RNA. As expected, unlabelled RNA from the 18-h aged tissue competed with [$^{32}$P]RNA from the same tissue very efficiently, while the competition by RNA from the tissue aged for 6 h was significantly smaller (Fig. 3). RNA from dormant tissue competed to a much lesser extent with the RNA from the 18-h tissue. From the competition curves one can estimate the percentage of internal homology between the investigated RNA populations. Rough calculations indicate that the "early" RNA (6 h) population contains as much as 40 - 45% of transcripts still present in the "late" (18 h) RNA. Homologous RNA copies extracted from dormant tissue compete with "late" RNA to about 15 - 20%. These data illustrate the degree of relatedness in RNA populations produced during tissue ageing.


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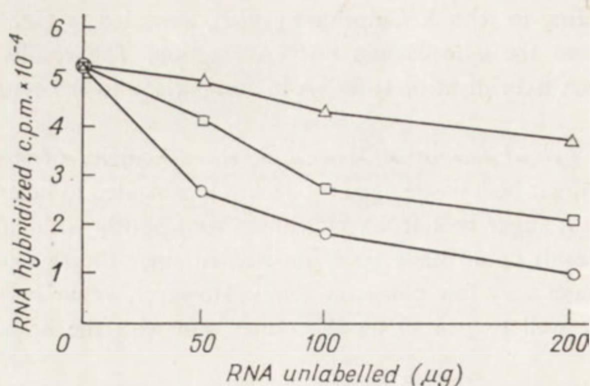


Fig. 3. Competition by unlabelled RNA isolated from sugar beet tissue at different stages of ageing in the hybridization of $^{32}$P-labelled RNA from 18-h aged tissue to homologous DNA. Fifty μg of [$^{32}$P]RNA (spec. act. 49 000 c.p.m./μg) from sugar beet aged for 18 h was hybridized to DNA in the presence of unlabelled RNA isolated from tissue: dormant (△); aged for 6 h (□); aged for 18 h (○). Annealing was carried out in 0.5 ml of 4×SSC at 65°C for 18 h.

## DISCUSSION

It is commonly accepted that differentiation consists in sequential derepression of structural genes, allowing for the transcription of specific informational RNA molecules which serve as templates for synthesis of new proteins. Tissues from roots and tubers provide an unique biological system for studying the transcriptional regulation of protein production in plants. A number of enzymes present at very low levels or absent from dormant tissue begin to appear and increase when sliced tissue is aerated in buffered solutions (Edelman & Hall, 1965; Leaver, 1966; Glasziou *et al.*, 1967; Cherry, 1968). The effect of ageing of sugar beet tissue on the enhancement of invertase activity has been thoroughly studied (Cherry, 1968). It was found that the enzyme is synthesized *de novo* and requires RNA synthesis since actinomycin D virtually arrested invertase production. Further studies on RNA synthesis during the first 18 h of tissue ageing showed a large increase in RNA production, the rapidly labelled RNA fraction included (Wasilewska & Cherry, 1974). Thus it might be expected that the overall enhancement of RNA synthesis would be accompanied by changes in the degree of heterogeneity of the RNA population. The results of our RNA - DNA hybridization studies confirmed this supposition and showed that at particular stages of tissue ageing distinct changes occurred in the types of RNA synthesized, while certain RNA species were produced throughout the whole period of tissue development. This indicates that during 0 - 18 h ageing of sugar beet tissue more and more genes become switched on, allowing for synthesis of RNA enriched in new types of transcripts, similarly as it was demonstrated during the first three hours of mouse liver regeneration (Church & McCarthy, 1967).

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Since rRNA and tRNA are not expected to contribute significantly to changes in the degree of RNA heterogeneity, the observed production of new transcripts with tissue ageing is thought to concern mostly the messenger and/or heterogeneous nuclear RNAs.

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## HYBRYDYZACJA ZNAKOWANEGO RNA DO DNA Z KORZENIA BURAKA CUKROWEGO

Streszczenie

1. Wykazano, że populacja RNA syntetyzowanego podczas pierwszych 18 godz. po przerwaniu stanu spoczynku korzenia buraka cukrowego podlega zmianom jakościowym.

2. Wyniki hybrydyzacji kompetycyjnej wskazują, że populacja „późnego" (18 godz.) RNA zawiera około 40% „wczesnych" (6 godz.) transkryptów RNA. Na podstawie otrzymanych wyników sugeruje się, że za zmianę stopnia heterogenności RNA odpowiedzialna jest synteza bądź różnych gatunków mRNA, bądź też specyficzne RNA jądrowe.

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# ISOLATION OF IgA AND OF A FAST SUBFRACTION OF IgG FROM HUMAN PLACENTA EXTRACT

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Placental extract was fractionated with ethanol and chromatographed on DEAE-Sephadex column, pH 6.3. A fraction was obtained consisting of IgA and IgG. The two immunoglobulins were separated using immobilized anti-human $\gamma$-chain antibody, and were further purified by gel-chromatography. IgA and IgG were obtained free of other immunoglobulin classes and serum proteins. The isolated IgA exhibited typical properties of serum IgA. IgG subfraction had the electrophoretic mobility equal to that of IgA, its hexose content was 11 moles per mole protein, the sedimentation coefficient, $s^0_{20,w}$ was 6.80 S, and the molecular weights of its light and heavy chains were 23 000 and 45 000, respectively.

Class A immunoglobulin (IgA) constitutes only about 10% of total immunoglobulin present in normal human serum. Low concentration and solubility characteristics make the purification of IgA particularly difficult. Consequently, most of the properties of IgA were investigated using myeloma proteins or secretory immunoglobulin rather than those isolated from normal serum.

The earlier procedures for the isolation of IgA included numerous fractionation steps, and relied for the final purification on zonal electrophoresis (Schultze & Heremans, 1966). The yield was low, and the product obtained may not have been well representative of the normal spectrum of serum IgA because only the fraction was isolated of the highest electrophoretic mobility (Heremans *et al.*, 1965). A particularly tenacious contamination of IgA consisted of a subfraction of IgG which had a similar electrophoretic mobility. This IgG was removed by Zschocke *et al.* (1969) using immunosorption but, again, after a multistage fractionation. The yield of IgA was low, and the properties of the fast IgG were not investigated.

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During the large-scale fractionation of human plasma with ethanol, IgA is separated from the predominant fraction of IgG, and is found mainly in Cohn's Fraction III. A preparation rich in IgA but containing some 10% of IgG was obtained from Fraction III by Steinbuch (1964).

In the present work we describe a procedure for an efficient recovery of IgA and of a fast subfraction of IgG from human placental extract.

## MATERIALS AND METHODS

*Immunoglobulins* were isolated from Fraction B-1 supplied by the gamma-globulin production department of Biomed (Lublin, Poland). It was shipped as a deep-frozen paste, and has been stored at -10°C until used. The composition of Fraction B-1 is quite similar to that of Cohn's Fraction III obtainable from human serum, but it contains more haemoproteins and some proteins of tissue origin.

*Immune sera.* Monospecific immune sera against human $\gamma$, $\alpha$, $\mu$, $\delta$, and $\varepsilon$ chains were purchased from Behringwerke (Mahrburg, G.F.R.). In some experiments, anti-human IgG and anti-human IgA sera produced by Biomed, Warsaw, were used. From this last source were also obtained goat anti-human Fc and equine anti-human serum proteins (anti-human) serum.

*Other proteins.* For the calibration of Sephadex columns and of polyacrylamide gels, the following proteins were used: human gamma-globulin (mol. wt. 160 000) and human serum albumin (mol. wt. 66 000) purchased from Biomed (Warszawa, Poland); porcine pepsin, cryst. (mol.wt. 35 000), a product of Worthington Biochem. Corp. (Freehold, N.J., U.S.A.); carboxypeptidase, cryst. (mol.wt. 34 600), a Reanal (Budapest, Hungary) product; chymotrypsinogen, cryst. (mol.wt. 25 700), a Sigma Chem. Corp. (St. Louis, Mo., U.S.A.) product; and cytochrome *c* (mol.wt. 11 000) produced by Biomed (Kraków, Poland).

*Other chemicals.* Sepharose, Sephadex and its derivatives were Pharmacia (Uppsala, Sweden) products. Other reagents were purchased from P.O.Ch. (Gliwice, Poland) and were used without further purification, except for urea which was crystallized from ethanol prior to use.

*Protein content* was determined by the biuret method (Hinsberg & Lang, 1957) with a purified human serum albumin preparation as the reference protein. When spectrophotometric measurements were employed, the value of $E^{1\%}_{280nm} = 13.6$ was assumed for all immunoglobulins.

*Hexose content* was determined by orcinol method (Winzler, 1955) with galactose as the reference sugar.

*Ultracentrifugation* was performed in Beckman L3-50 instrument with the Schlieren attachment, at 50 000 r.p.m. and at 20°C.

*Immunodiffusion and immunoelectrophoresis.* A 1.5% solution of agar in pH 8.6 barbital buffer was used throughout. Electrophoresis was carried out at 2.5 mA/cm at room temperature for two hours. Radial immunodiffusion was performed as described by Mancini *et al.* (1965) with several dilutions of appropriate standards included in each plate. All agar plates with the developed precipitation lines were

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washed exhaustively with 0.15 M-sodium chloride, stained with 0.05% Amido Black in 0.1 M-acetate buffer, pH 5.0, destained with 2% (v/v) acetic acid, and air-dried.

*SDS-polyacrylamide electrophoresis* was performed as described by Dunker & Rueckert (1969) but in a flat glass-sheet apparatus. Proteins were reduced with 1% mercaptoethanol in urea-containing buffer, and alkylated with excess iodoacetamide essentially as described by Weber & Osborn (1969). The gels were stained with 0.25% Coomassie Blue in 50% (v/v) aqueous solution of methanol containing 7% (v/v) acetic acid. The same solvent was used for destaining. Calibrating proteins were included in each plate.

*Affinity chromatography*. Proteins were coupled with Sepharose 6B activated with CNBr (Cuatrecasas *et al.*, 1968), using 3 mg of protein per 1 ml of Sepharose. The following immobilized proteins were prepared for the present investigation: human immunoglobulin light chains, human immunoglobulin $\gamma$-chains, and rabbit antibodies against human $\gamma$-chain. Commercial preparation of gamma-globulin was a starting material for the preparation of immunoglobulin chains as well as for the immunization of animals. Gamma-globulin was purified by DEAE-chromatography, as described under Results, and the fraction which was not retained by the column was used to prepare the immunoglobulin chains. It was reduced with mercaptoethanol as described by Fleischman *et al.* (1962) and the polypeptide chains were separated by Sephadex G-200 chromatography in 0.5 M-acetic acid.

In order to produce anti-IgG serum, rabbits were immunized with weekly injections of purified gamma-globulin, 5 mg, emulsified with incomplete Freund adjuvant, per dose. When a high titre antibody was produced, the rabbits were bled and the serum was passed through a column with immobilized human immunoglobulin light chains to remove anti-L antibodies. Anti-$\gamma$ antibody was then extracted using a column with immobilized human $\gamma$-chains. Antibodies were dissociated from the sorbent with 4 M-potassium thiocyanate adjusted to pH 5.0. After the thiocyanate was removed by dialysis and the protein concentrated by ultrafiltıation, the antibodies weıe coupled with Sepharose.

The capacity of all immobilized proteins was approximately 1 mg per ml of Sepharose. To regenerate the immunosorbents, the columns were washed with 6 M-urea in 0.1 M-hydrochloric acid followed by 0.15 M-sodium chloride containing 0.2 M-sodium azide. Under these conditions, the sorbents could be recycled at least ten times without an appreciable loss of capacity.

## RESULTS

Fraction B-1 contains an appreciable amount of denatured lipoproteins which form an intractable suspension in aqueous solvents. This insoluble material had to be removed prior to further fractionation (cf. Table 1). Each 100 g of B-1 paste, still partially frozen, was blended with ice-cold 0.15 M-sodium chloride, pH 7.0. The suspension was stirred at 4°C for three hours, after which 95% (v/v) ethanol pre-cooled to $-20$°C was slowly added to bring the final concentration of ethanol

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to 10% (v/v). During the addition the suspension was well stirred, and its temperature was gradually lowered to −3°C. At this temperature it was left overnight. The next day the precipitate was removed by centrifugation at 2500 *g* at −3°C and the concentration of ethanol in the supernatant was increased to 30% (v/v) with the precooled 95% ethanol. During the addition of ethanol the suspension was stirred and its temperature was lowered to −6°C. It was left without stirring for about 18 h at −6°C, after which the precipitate was collected by 2500 *g* centrifugation at −6°C. The precipitate ("enriched immunoglobulin preparation") was either immediately processed or stored at −20°C. Agar immunodiffusion using monospecific sera showed the presence of IgG, IgA, IgM, and IgD but no IgE in the enriched immunoglobulin preparation. About 20% of IgA was lost during these initial steps in fractionation; other immunoglobulins were not quantitatively investigated.

Table 1

*Flow sheet — the isolation of IgA and of a fast IgG from the Fraction B-1 of human placenta extract*

| | |
|---|---|
| STEP 1: | *Fraction B*-1<br>Protein concentration 3%, 0.15 M-sodium chloride, pH 7.0, ethanol 10% (v/v), temp. —3°C, 18 h. |
| STEP 2: | *Supernatant B*-1*s*<br>Protein concentration 2.5%, pH 7.0, ethanol 30% (v/v), temperature —6°C, 18 h. |
| STEP 3: | *Precipitate* 2 (*Enriched immunoglobulin preparation*)<br>0.0175 M-phosphate buffer, pH 6.3, Sephadex A-50 column chromatography, fraction selected between 0.2 and 0.3 M-sodium chloride. |
| STEP 4: | *Peak II* (*IgA-IgG*)<br>Affinity chromatography on immobilized rabbit anti-human γ-chain antibody. |
| STEP 5: | *Non-retained and retained protein fractions*<br>Sephadex G-200 chromatography, 0.15 M-sodium chloride, pH 7.0, fraction selected at mol. wt. about 160 000. Monomeric IgA thus isolated from the non-retained fraction, and monomeric IgG from the retained fraction. |

The enriched immunoglobulin preparation was dissolved in 0.15 M-sodium chloride, pH 7.0, and exhaustively dialysed against 0.0175 M-phosphate buffer, pH 6.3. The solution was then applied on a column of DEAE-Sephadex A-50 equilibrated with the same buffer. After the first unretained peak emerged (Fig. 1), and the extinction dropped to low values, the column was eluted successively with 0.1, 0.2, 0.3, and 0.5 M-sodium chloride in the same pH 6.3 phosphate buffer. Five well separated peaks were thus obtained. Slightly below 80% of the total protein was recovered from the column, and about 70% of IgA. Nearly two-thirds of all IgA was found in Peak II (cf. Table 2).

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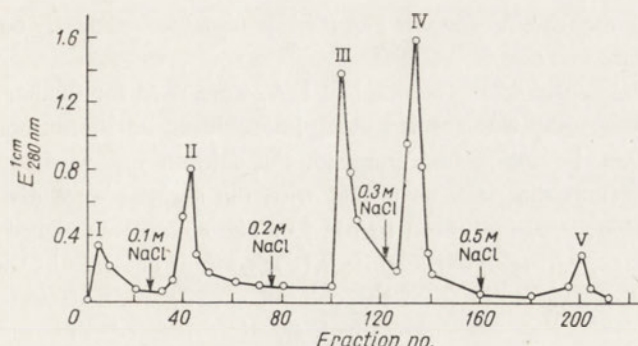


Fig. 1. DEAE-Sephadex A-50 chromatography of the enriched immunoglobulin preparation. Column 2.5×90 cm, 0.0175 M-phosphate buffer, pH 6.3, eluted with the same buffer but of increased molarity of sodium chloride as indicated with the arrows. Protein load 775 mg. Fractions, 5.0 ml, collected at a rate of 40 ml/h.

Table 2

*Distribution of IgA and IgG among the fractions eluted from Sephadex A-50 column*

| Fraction[a] | IgA[b] (mg) | Relative amount per fraction (%) | | |
|---|---|---|---|---|
| | | IgA | IgG | other protein |
| Peak I | 0 | 0 | 100 | 0 |
| Peak II | 24 | 36 | 63 | 1 |
| Peak III | 11 | 12 | 19 | 70 |
| Peak IV | 3 | 8 | 2 | 90 |
| Peak V | 0 | 0 | 0 | 100 |

[a]Cf. Fig. 1.
[b]Column loaded with 775 mg protein containing 54 mg IgA.

Monospecific sera detected the presence of IgG only in Peak I, while IgG, IgA, and IgM were found in Peaks II, III, and IV; Peak V was not examined. IgD could not be detected in any of the fractions eluted from DEAE-Sephadex column, despite its presence in the material applied on the column. Immunoelectrophoretic patterns developed with anti-human serum are depicted in Plate 1. A single sharp precipitation arc was produced by Peak I protein in the far cathodic region. In Peak II, a single but less homogeneous and of a somewhat faster mobility arc is apparent. On freezing and thawing of this fraction, a double precipitation arc was formed (cf. Plate 3). Two relatively fast-moving proteins were found in Peak III, and a number of faint precipitation lines in Peak IV.

Table 2 shows the distribution of IgA and IgG among the fractions eluted from DEAE-Sephadex column, as determined by radial immunodiffusion. Within the limits of the experimental error, all protein contained in Peak I could be accounted for by IgG, and those in Peak II by the sum of IgG and IgA. Proteins which were neither IgA nor IgG constituted most of the remaining fractions. The IgA:IgG ratio in Peak II varied, probably depending on the composition of Fraction B-1,

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and occasionally the fraction obtained as Peak II consisted of nearly equal amounts of IgA and IgG.

Peak II proteins, the most abundant in IgA, were used for further purification. Affinity chromatography was applied using immobilized anti-$\gamma$ antibodies (Fig. 2). After the unretained fraction had emerged, the column was washed with 0.15 M-sodium chloride, and the proteins bound with the sorbent were dissociated with 4 M-potassium thiocyanate adjusted to pH 5.0. The eluate was placed immediately in cellophane bags, and dialysed against 0.15 M-sodium chloride in the cold-room. The recovery of total protein from the column was between 90 and 100 percent. In agar immunodiffusion, the unretained fraction reacted with anti-$\alpha$ but not with anti-$\gamma$ serum (Plate 2), and usually produced a faint precipitation line with anti-$\mu$ serum. The retained fraction was precipitated by anti-$\gamma$ serum (Plate 2) but not with anti-$\alpha$ and anti-$\mu$ serum.

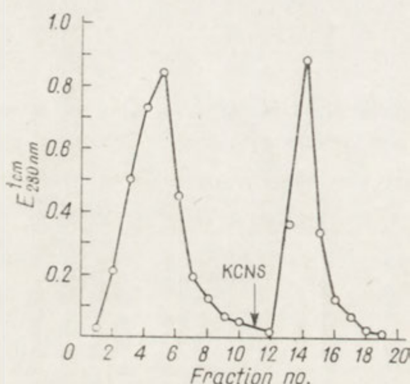


Fig. 2. Affinity chromatography of Sephadex A-50 Peak II proteins. Column 2.5×7 cm filled with immobilized rabbit anti-human $\gamma$-chain antibody, 1 mg per ml of Sepharose. Total protein applied 20 mg. The arrow indicates the start of the elution with 4 M-potassium thiocyanate, pH 5.0.

The two fractions obtained from the affinity column were subjected to gel chromatography on a calibrated Sephadex G-200 column in 0.15 M-sodium chloride, pH 7.0 (Fig. 3). The major peak isolated from the unretained fraction (Fig. 3A) emerged in a volume indicating its molecular weight to be 170 000, as expected for the monomeric IgA. A small peak eluted with the void volume contained, presumably, aggregated IgA and IgM, while the last peak, mol. wt. 11 700, did not produce a stainable band in polyacrylamide electrophoresis, and was not further examined. Immunoelectrophoretic patterns (Plate 3) showed that the molecular sieve chromatography removed the high molecular weight proteins from the IgA fraction.

The other fraction obtained from the affinity column also produced three peaks in the Sephadex G-200 elution profile (Fig. 3B) but here the magnitude of the first, unretarded, peak was nearly equal to that of the second peak which was eluted with mol. wt. 160 000, as expected for a monomeric IgG. The third peak appeared similar to the corresponding peak found in the Sephadex eluate of IgA-containing fraction, and was not further investigated. Immunoelectrophoretic analysis (Plate 4) showed two parallel precipitation arcs of equal mobility in the thiocyanate eluate prior to Sephadex G-200 chromatography. As it is shown in Plate 4, one of these

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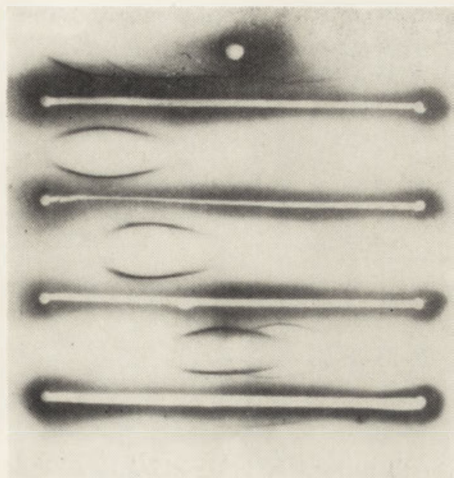

Plate 1. Immunoelectrophoresis of proteins eluted from Sephadex A-50 column. Troughs: equine anti-human serum. Wells, top to bottom: enriched immunoglobulin preparation, 27 mg/ml; Peak I, 5 mg/ml; Peak II, 5 mg/ml; Peak III, 7 mg/ml; Peak IV, 1.5 mg/ml. Cf. Table 1 and Fig. 1.

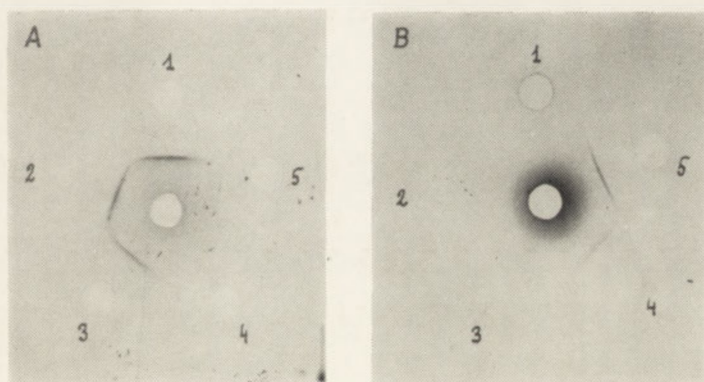


Plate 2. Immunodiffusion patterns of proteins separated by affinity chromatography. Centre well: *A*, anti-$\gamma$ serum; *B*, anti-$\alpha$ serum. Outer wells: *1*, proteins dissociated from the immunosorbent with potassium thiocyanate; *2*, polymeric IgG isolated by Sephadex G-200 chromatography from the fraction retained by the immunosorbent; *3*, monomeric IgG (cf. Fig. 3B); *4*, protein non-retained by the immunosorbent; *5*, monomeric IgA (cf. Fig. 3A).



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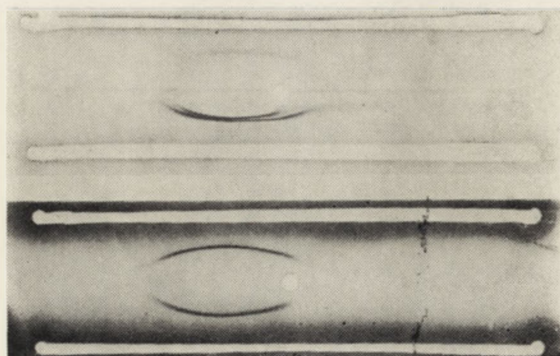

Plate 3. Immunoelectrophoresis patterns of IgA purified by Sephadex G-200 chromatography. Top: protein applied on Sephadex column; anti-α serum in the upper trough, and anti-human serum in the lower trough. Bottom: monomeric IgA; anti-human serum in both troughs.

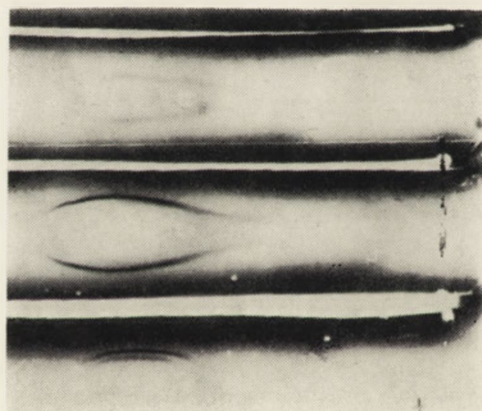

Plate 4. Immunoelectrophoresis patterns of IgG purified by Sephadex G-200 chromatography. Anti-human serum in all troughs. Bottom to top: unfractionated KCNS-eluate; monomeric fraction; polymeric fraction.



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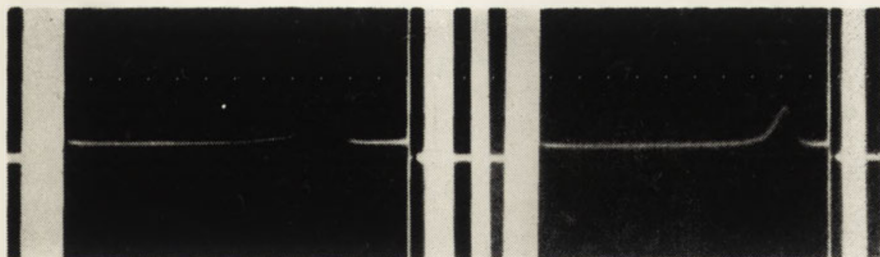

Plate 5 Plate 6

Plate 5. Sedimentation velocity pattern of monomeric IgA. Protein concentration 1.0 mg/ml, in 0.15 M-sodium chloride, pH 7.0. The photograph was taken 40 min after the rotor reached 50 000 r.p.m. Temp. 20°C. Migration from left to right.

Plate 6. Sedimentation velocity pattern of monomeric IgG. Protein concentration 0.8 mg/ml, in 0.15 M-sodium chloride, pH 7.0. The photograph was taken 20 min after the rotor reached 50 000 r.p.m. Temp. 20°C. Migration from left to right.

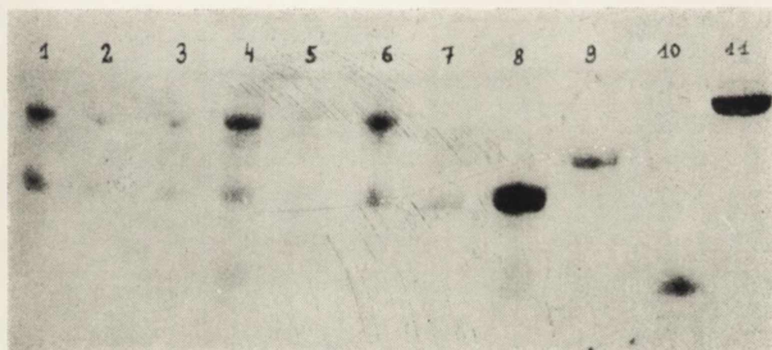


Plate 7. SDS-polyacrylamide gel electrophoresis of reduced and alkylated immunoglobulins. From the left: *1*, IgA; *2* and *3*, IgG; *4*, Peak II from Sephadex A-50 column (cf. Fig. 1); *5*, IgA; *6*, IgG; and the calibrating proteins: *7*, chymotrypsinogen; *8*, carboxypeptidase; *9*, pepsin; *10*, cytochrome *c*; *11*, human serum albumin.



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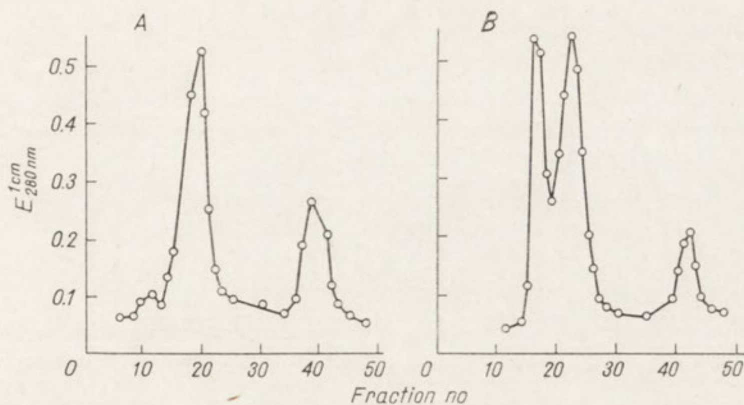


Fig. 3. Chromatography on Sephadex G-200 column of proteins resolved by affinity chromatography. Column 2.5×90 cm, eluted with 0.15 M-sodium chloride, pH 7.0, at a rate of 20 ml/h, 2.5 ml fractions collected. *A*, non-retained fraction; *B*, retained fraction (cf. Fig. 2).

arcs was recovered in the first, and the other in the second peak eluted from Sephadex G-200 column. When these peaks were analysed by agar immunodiffusion (Plate 2), both reacted with anti-$\gamma$ but not with anti-$\alpha$ serum. Thus, the heavy molecular weight material consisted of aggregated IgG.

Monomeric IgA produced a single and apparently symmetrical peak in the ultracentrifuge diagram (Plate 5). The relationship between the concentration and the sedimentation coefficients was linear (Fig. 4). The sedimentation constant extrapolated to zero concentration and appropriately corrected, $s^{0}_{20,w}$, was 6.98 S. Hexose content was 29.1 moles per 170 000 g. Reduced and alkylated IgA yielded two bands in SDS-polyacrylamide electrophoresis (Plate 6), of molecular weight 55 000 for the heavy chain, and 23 000 for the light chain.

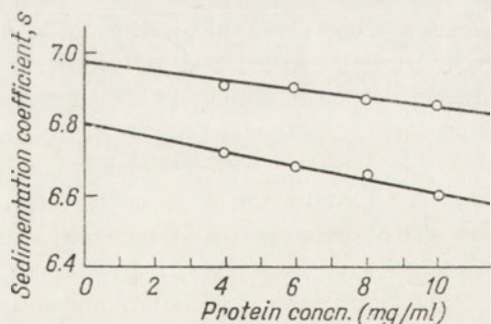


Fig. 4. Dependence of sedimentation coefficients on the concentration of immunoglobulin. Upper plot: IgA. Lower plot: IgG.

Monomeric IgG also appeared homogeneous in the ultracentrifuge analysis (Plate 7). The sedimentation coefficient, extrapolated from the linear plot against the concentration (Fig. 4), $s^{0}_{20,w}$, was 6.80 S. IgG contained 11.4 moles of hexose per 160 000 g. The molecular weight of the polypeptide chains, calculated from SDS-polyacrylamide electrophoresis (Plate 6) was 45 000 and 23 000 for the heavy and the light chain, respectively.

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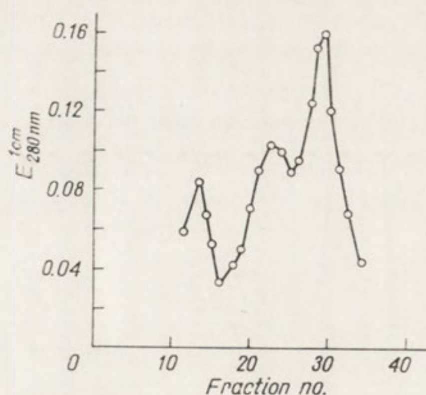


Fig. 5. Resolution of tryptic digestion products of fast IgG subfraction on Sephadex G-200 column. Conditions of chromatography as in Fig. 3. Protein load, 30 mg.

Susceptibility of the isolated monomeric IgG to tryptic digestion was investigated under the conditions described by Gergely *et al.* (1972). IgG was treated with trypsin at the enzyme: protein weight ratio of 1:25 in pH 8.1 phosphate buffer for 18 h at 37°C. The digest was chromatographed on G-200 Sephadex column in pH 7.0 sodium chloride, 0.15 M. Three peaks were obtained (Fig. 5), the first close to the void volume, the second of mol. wt. 102 000, and the third with mol. wt. 39 000. Thus, the tryptic digestion resulted in the formation of $F(ab)_2$ fragment and Fc fragment. The presence of Fc was confirmed by its reaction with anti-human Fc serum in agar immunodiffusion.

## DISCUSSION

The absorption of IgA by DEAE-sorbents is not considered remarkably selective. In the methods elaborated for the purification of immunoglobulins these ion-exchangers are usually employed batchwise for a preliminary enrichment step. Recently, Litman & Good (1972) obtained from DEAE-cellulose column a fraction of human serum which consisted almost exclusively of IgA. In the present work, DEAE-Sephadex chromatography yielded a fraction which comprised between 50 and 70 percent IgG, the rest being IgA, with other serum proteins practically absent. While the amount of non-immunoglobulin proteins in a narrowly cut fraction was similar to what Litman & Good reported, the relative amounts of IgA and IgG were widely discrepant. As the conditions of chromatography were not too dissimilar, and with the placenta extract consisting almost only of serum proteins, the discrepancy probably reflects the difference between the composition of the initial material, serum in one case, and placental fraction B-1 in the other.

It seemed unlikely that IgA could be separated from the accompanying IgG by conventional methods of fractionation, since the two proteins exhibited similar electrophoretic and chromatographic properties. Affinity chromatography, on the other hand, appeared to be the method of choice but the abundance of IgG made it necessary to prepare an immunosorbent of high capacity. This was achieved by coupling with Sepharose the purified anti-$\gamma$ antibody instead of anti-IgG serum

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globulins, as used by Zschocke *et al.* (1969). IgA could also be extracted directly by means of immobilized anti-$\alpha$ chain antibody. This approach is useful for a rapid isolation of small quantities of IgA (Anderson *et al.*, 1970) but it was not considered suitable in the investigation aimed at the isolation of non-denatured IgA, because irreversible aggregation always accompanied the dissociation of immunoglobulin from the sorbent. The reverse procedure, as employed in this work, does not affect the properties of IgA nor does it cause an appreciable loss of IgA during this critical step in the purification procedure. The over-all yield of IgA was about 35%, thus making it possible to obtain about 0.5 g of IgA from 1000 g (wet weight) of Fraction B-1. The loss of IgG, mainly at the affinity chromatography step, was larger so that the final yield of the IgG-subfraction was also about 0.5 g per 1000 g of the B-1 paste (Table 3).

Table 3

*The yield of IgA and of IgG from Fraction B-1*

| Fractionation step[a] | Yield, grams per 1000 g, Fraction B-1[b] | |
|---|---|---|
| | IgA | IgG |
| Step 2, ethanol extract | 1.56 | —[c] |
| Step 3, DEAE-Sephadex, Peak II | 0.68 | 1.20 |
| Step 5, monomeric protein | 0.45 | 0.48 |

[a]Cf. Table 1.
[b]Wet weight.
[c]Contains also slow IgG subfraction(s).

The isolated IgA had the molecular weight, hexose content, and the molecular weight of its heavy chain typical for class A immunoglobulins. Both the sedimentation coefficient and Sephadex elution volume show that only the monomeric IgA was extracted. The polymerized IgA may have been removed during DEAE-chromatography, but it seems that Fraction B-1 was already less abundant in this IgA-subfraction.

IgG which was eluted from the immunosorbent was resolved by Sephadex G-200 chromatography into two fractions. The heavy molecular weight protein was IgG by serological criterion, and its state of aggregation presumably resulted from the dissociation of the antigen-antibody complex. The monomeric IgG had the sedimentation coefficient, hexose content, and the heavy-chain molecular weight typical for its class, but it differed from the predominant fraction of serum IgGs by its fast electrophoretic mobility. Abel *et al.* (1968) have found, among a large number of myeloma IgGs, about 30 percent with the hexose content within the range 19 to 37 moles per mole protein. High carbohydrate content in immunoglobulin is correlated with a fast electrophoretic mobility, but the IgG subfraction isolated in this work has a low hexose content while exhibiting a fast mobility. The recognized

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subclasses of IgG are detected in immunoelectrophoretic patterns of human serum as proteins of various electrophoretic mobilities. However, under the conditions of DEAE chromatography, the subclasses (except perhaps for IgG4) are distributed among the retained and the unretained subfractions of IgG (Skvaril & Morell, 1970), and were shown to have the electrophoretic mobility characteristic for the chromatographically defined subfraction rather than for the specific antigenic marker. In addition to the heterogeneity of electrophoretic mobility, hexose content, and chromatographic properties, the myeloma proteins which belong to different subclasses of IgG were found to differ in their biological properties. The IgG2 subclass was not fixed in skin tissue (Terry, 1965), and was quite resistant to proteolytic digestion (Gergely *et al.*, 1972). The IgG4 myeloma protein was not able to bind complement (Ishizaka *et al.*, 1967), and did not produce the Fc fragment on tryptic digestion (Gergely *et al.*, 1972). The pattern of tryptic digestion products of the isolated fast IgG subfraction was normal in that both $F(ab)_2$ and Fc fragments were present. Preliminary experiments showed that it was fixed in the guinea pig skin, under the conditions of reverse passive anaphylaxis test, and that its complement-fixing ability was equal to that of the bulk of serum IgG. Thus the isolated fast subfraction of IgG does not share the biological properties with some selected pathological subfractions of IgG.

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IZOLOWANIE IgA ORAZ SZYBKIEJ SUBFRAKCJI IgG Z EKSTRAKTU ŁOŻYSKOWEGO

Streszczenie

Wyciąg łożyskowy frakcjonowano etanolem i poddano go chromatografii na kolumnie z DEAE-sefadeksem w pH 6.3. Otrzymano frakcję złożoną z IgA i IgG. Dwie te immunoglobuliny rozdzielono stosując immobilizowane przeciwciała swoiste dla ludzkich łańcuchów $\gamma$, a następnie oczyszczono je za pomocą chromatografii żelowej. Otrzymano IgA oraz IgG wolne od innych immunoglobulin i białek surowicy. IgA posiadały typowe właściwości monomerycznych IgA surowicy. Wyizolowane IgG wykazywały wysoką ruchliwość elektroforetyczną, równą ruchliwości IgA, zawierały 11 moli heksoz na mol białka, ich stała sedymentacji, $s^0_{20,w}$, wynosiła 6.80 S, c.cz. łańcucha lekkiego był 23 000, a ciężkiego — 45 000.

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# ACID PHOSPHATASE FROM HUMAN PROSTATE
## CHEMICAL MODIFICATION AND ACTIVITY OF THE ENZYME

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Iodine monochloride (ICl) at a 70-fold molar excess and *N*-bromosuccinimide (NBS) at a 50-fold molar excess completely inhibited the prostatic phosphatase activity; tetranitromethane (TNM) at a 300-fold molar excess inhibited the enzyme only by 40%. ICl and TNM reacted mainly with tyrosine residues; NBS oxidized mainly tryptophan. The modified enzyme dissociated into inactive subunits (52 000 - 55 000 daltons). Fluorescence intensity of the modified enzyme markedly decreased in the region characteristic for exposed tryptophan residues. The phosphatase was readily photoinactivated in the presence of Rose Bengal as sensitizing dye. The rate of inactivation was first order, and it was much lower in the presence of substrates or competitive inhibitors. The photoinactivation rate was constant in the pH range 4.8 - 6.0 and increased rapidly at higher pH values, paralleling the theoretical ionization curve of imidazole. Photoinactivation was accompanied by modification of about 14 histidine residues but no dissociation of the enzyme into subunits was observed. Analysis of the results indicates that histidine residues probably take part in the active site of prostatic phosphatase, whereas tyrosine and tryptophan residues are not directly involved in the catalytic function of this enzyme.

Earlier studies in this laboratory on the mechanism of catalysis of human prostatic acid phosphatase (EC 3.1.3.2) showed that iodination (Bobrzecka *et al.*, 1968) and oxidation with NBS (Bobrzecka *et al.*, 1969) inactivate the enzyme. The involvement of histidine in formation of phosphoryl-enzyme intermediate of the acid phosphatase from human prostate (Ostrowski & Barnard, 1973) and other sources (Feldman & Butler, 1969; Igarashi *et al.*, 1970; Hickey & VanEtten, 1972) was postulated on the basis of investigations of hydrolysis and transphosphorylation reactions.

To obtain more information on the role of cyclic amino acid residues in catalytic function of prostatic phosphatase, the enzyme was modified by iodination, nitration, oxidation or photooxidation, and the changes in enzymic activity, protein fluorescence intensity and dissociation into subunits were investigated.


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## MATERIAL AND METHODS

Acid phosphatase from hypertrophic human prostate glands was prepared as described previously (Ostrowski, 1968). The obtained enzyme was homogeneous when examined by several methods (Ostrowski, 1968; Derechin *et al.*, 1971).

Disodium salt of pNPP[1], $\beta$-glycerophosphate, and 3′-UMP were from Koch-Light (Colnbrook, Bucks., England); Rose Bengal, containing 84% of pure dye, was from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.); disodium phenyl-phosphate, sodium creatine phosphate and 5′-AMP were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Phosphoethanolamine and TNM were from Aldrich Chem. Corp. (Milwaukee, Wis., U.S.A.); L(+)-tartrate, papain, cryst. and bovine serum albumin, cryst. were fiom B.D.H., Inc. (Poole, England); pepsin was from N.B.C. (Cleveland, Ohio, U.S.A.); cytochrome *c*, from Reanal (Budapest, Hungary). Sephadex G-25, medium and Sephadex G-200, fine, were from Pharmacia (Uppsala, Sweden). Other reagents were of analytical grade.

Radioactive iodine monochloride, $^{131}$ICl (spec. act. 3.5 μCi/ml), was obtained according to Izzo *et al.* (1958) from Na$^{131}$ICl of spec. act. 17.9 μCi/ml, which was supplied by the Isotope Distribution Division (Warszawa, Poland). NBS was prepaıed as described by Vogel (1964).

*Enzymic activity* of acid phosphatase was measured with 0.02 M-solution of pNPP in 0.05 M-citrate buffer, pH 5.0, under conditions described previously (Ostrowski & Tsugita, 1961).

*Iodination of acid phosphatase.* The enzyme preparation was iodinated by $^{131}$ICl as described by Bobrzecka *et al.* (1968). The reaction was stopped by adding an excess of $Na_2S_2O_3$ to reduce the unutilized iodine. Then 6 mg of crystalline bovine serum albumin per ml was added, and the mixture dialysed against 0.9% NaCl for over 100 h, with repeated changes of the external solution. Radioactivity of the sample was measured with a scintillation counter US-2 (INR, Warsaw-Świerk, Poland). The blank contained Na$^{131}$I instead of $^{131}$ICl.

*Nitration of phosphatase.* The enzyme was nitrated according to the method described by Riordan *et al.* (1967). To a solution of 1 - 2 nmol of enzyme protein in 0.5 ml of 0.05 M-Tris-HCl buffer, pH 8.0, TNM (10% solution in 95% ethanol) was added to a TNM : protein molar ratio ranging from 100 to 6000, and the reaction was allowed to proceed at 20°C for 1 - 1.5 h. After exhaustive dialysis against the above buffer, the nitrotyrosyl content was determined at 427 nm, using for calculation $\varepsilon_{427nm}^{pH8.0} = 3800$ (Riordan *et al.*, 1967).

*Reaction of phosphatase with NBS.* The reaction was carried out in 0.1 M-acetate buffer, pH 4.0 - 5.0, at room temperature. To 5 nmol of enzyme in 0.4 ml of buffer of appropriate pH, NBS (3 mM solution in the same buffer) was added to the required concentration. The amount of oxidized tryptophan residues was determined at 280 nm, using $\varepsilon_{280nm}^{pH4.5} = 5500$ (Patchornik *et al.*, 1958).

[1] Abbreviations: TNM, tetranitromethane; NBS, *N*-bromosuccinimide; pNPP, *p*-nitrophenyl-phosphate.

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*Photochemical oxidation of phosphatase.* This was performed with Rose Bengal as sensitizing dye by a modification of the procedure of Westhead (1965). The reaction was carried out in 0.1 M-Tris-HCl buffer, pH 7.4, containing 1 mM-EDTA, 0.1 mg/ml of Rose Bengal and 50 μg/ml of phosphatase protein. The sample was incubated at 20°C in 2-ml spectrophotometric cell using two 300-W slide projectors as light source at the distance of 30 cm. At different time intervals, 2-μl samples were withdrawn and the enzymic activity was determined.

*The molecular weight* determination of the modified phosphatase was accomplished by thin-layer gel filtration (Radola, 1968) on Sephadex G-200 plates (34 × 25.5 cm). The gel was swollen in 0.1 M-KCl - 0.05 M-Tris-HCl buffer, pH 6.5.

*Chemical determinations.* Tryptophan was determined according to Spies & Chambers (1949), histidine using amino acid analyser (Carlo Erba, A-23, Italy) and Pauly reaction (Macpherson, 1942). The protein content was measured spectrophotometrically, taking for calculation $E^{0.1\%}_{280\,nm} = 1.44$ (Bobrzecka *et al.*, 1968) and the molecular weight of the enzyme 102 000 daltons (Derechin *et al.*, 1971).

Absorption spectra were recorded on a Unicam SP-800 (England) spectrophotometer. Fluorescence measurements were made on a Fluorispec SF-1 spectrophotometer (Baird Atomic Inc., U.S.A.). pH was determined with a Ridan pH-meter (Unipan, Warszawa, Poland). Spectrophotometric titration was carried out using a small volume titration cell and "Agla" microburette as previously described (Bobrzecka *et al.*, 1968).

## RESULTS

*Iodination of phosphatase.* At a tenfold molar excess of $^{131}$ICl, no incorporation of iodine into the protein was observed (Fig. 1). At 25-fold excess of $^{131}$ICl, one atom of iodine was incorporated into the protein molecule, and the enzymic acti-

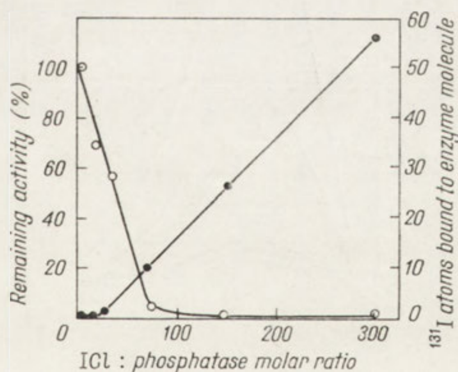


Fig. 1. Effect of molar excess of $^{131}$ICl on the activity of prostatic phosphatase (○), and binding of $^{131}$I atoms to the enzyme molecule (●). Samples of enzyme (0.5 mg protein in 1 ml of 0.05 M-Tris-HCl buffer, pH 8.1) to which 20 mM solution of $^{131}$ICl in 5 M-NaCl was added in appropriate amounts (final volume 1 ml), were incubated at 20°C for 3 min. Then 25 μl of $Na_2S_2O_3$ (1 μmol) was added and 10-μl portions were withdrawn and assayed for enzymic activity; to the remainder 6 mg of bovine serum albumin in 1 ml 0.9% NaCl was added, dialysed and radioactivity assayed in a scintillation counter.

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vity decreased by 35%. At higher molar ratios of $^{131}$ICl to the enzyme, the incorporation of iodine was linear, and at a 70-fold molar excess, about 10 atoms of iodine were incorporated per one molecule of phosphatase. At the same time, the enzymic activity almost disappeared. The enzyme completely blocked with iodine showed mono- and diiodo-derivatives of tyrosine when examined by spectrophotometric titration and by amino acid analysis of alkaline hydrolysate (Rybarska, 1970).

The course of the curve in Fig. 1 suggests that partial inactivation of the enzyme occurred even before incorporation of $^{131}$I into protein. At this stage the inactivation by iodine could be due to oxidation of sulphydryl groups (Fraenkel-Conrat, 1955) or tryptophan, histidine and methionine residues (Cha & Scheraga, 1963; Filmer & Koshland, 1964). The prostatic acid phosphatase contains two non-reactive SH groups (Domański *et al.*, 1964) but, as demonstrated by Bobrzecka *et al.* (1968), these groups were not oxidized by iodination. On the other hand, it was found by amino acid analysis that of the 18 tryptophans and 26 histidines present in the enzyme molecule (Derechin *et al.*, 1971), 1.3 and 2.4, respectively, could not be demonstrated in the phosphatase inactivated by a 70-fold excess of ICl.

Oxidation of tryptophan residues during iodination of phosphatase was followed by measurement of fluorescence emission spectra. The spectrum of the native phosphatase excited at 280 nm showed a maximum of the emission at 355 nm (Fig. 2), which was due to tryptophan residues accessible to the solvent (Brand & Witholt

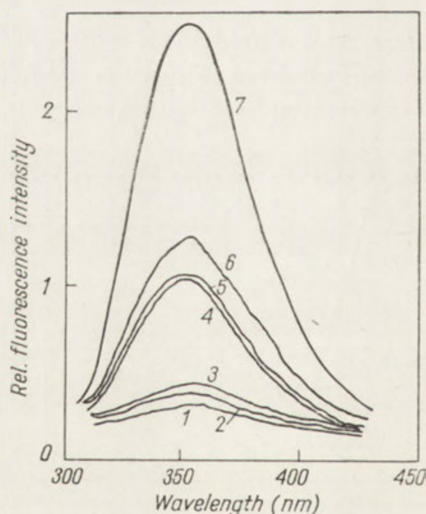


Fig. 2. Fluorescence emission spectra (uncorrected) of native and modified prostatic phosphatase. *1*, phosphatase oxidized with a 50-fold molar excess of NBS; *2*, iodinated with 70-fold molar excess of ICl; *3*, nitrated with 2000-fold molar excess of TNM; *4*, oxidized in the presence of 10 mM-5′-AMP; *5*, iodinated in the presence of 10 mM-pNPP; *6*, nitrated in the presence of 100 mM-L(+)-tartrate; *7*, native enzyme. All samples of phosphatase after the reaction were dialysed against bidistilled water for 48 h, adjusted to pH 6.5 and diluted to 15 μg protein/ml, and the fluorescence emission spectra determined; excitation at 280 nm.

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1967). After blocking of activity with ICl, the phosphatase exhibited a broad maximum in the same position as the native enzyme, but fluorescence intensity decreased by about 85%. Iodination of the enzyme in the presence of 10 mM-pNPP or 5'-AMP as substrate, or 100 mM-L(+)-tartrate as a competitive inhibitor, caused only a 50% decrease in the intensity of fluorescence, and 30% of the original activity was still present, as determined after filtration of the enzyme through the Sephadex G-25 column.

On thin-layer gel filtration the phosphatase inactivated with ICl gave an inactive spot with molecular weight of about 52 000 (Fig. 3). A part of the applied protein migrated with the front of the solvent showing a high-molecular-weight species as the result of aggregation. Under the same conditions the native phosphatase gave a single band with a molecular weight of about 100 000 daltons, in agreement with the results of sedimentation equilibrium analysis (Derechin *et al.*, 1971).

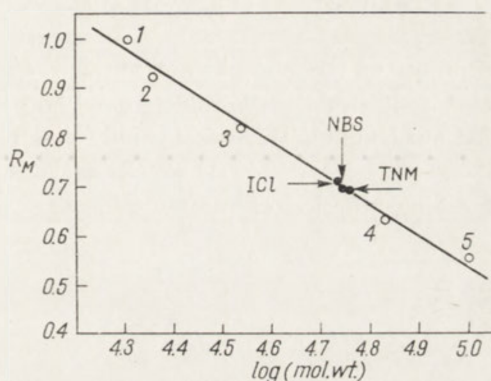


Fig. 3. Molecular weight determination of the modified phosphatase by thin-layer Sephadex G-200 gel filtration. The arrows show the position of acid phosphatase treated with a 70-fold molar excess of ICl, 2000-fold molar excess of TNM, and 50-fold molar excess of NBS. $R_M$ values are expressed as migration distances of proteins relative to migration distance of papain. Standard proteins: *1*, papain; *2*, cytochrome *c* dimer; *3*, pepsin, *4*, bovine serum albumin; *5*, native phosphatase.

From the above results it became clear that iodination, in addition to halogenation of tyrosine residues, caused partial oxidation of tryptophan and histidine residues and dissociation of the inactive prostatic phosphatase into its subunits.

*Nitration of phosphatase*. Preliminary experiments showed that the phosphatase was relatively resistant to nitration with TNM: under the applied conditions a very high excess of TNM was required. The relationship between the decrease in activity and the amount of TNM was biphasic (Fig. 4). At an about 300-fold molar excess of TNM to the enzyme, the activity was inhibited by about 40%, whereas an excess of even several thousands caused a further decrease in activity by only 20%.

The absorption and difference spectra of nitrated phosphatase (Fig. 5) showed a characteristic peak at 427 nm for nitrosyl chromophore (Vallee & Riordan, 1969).

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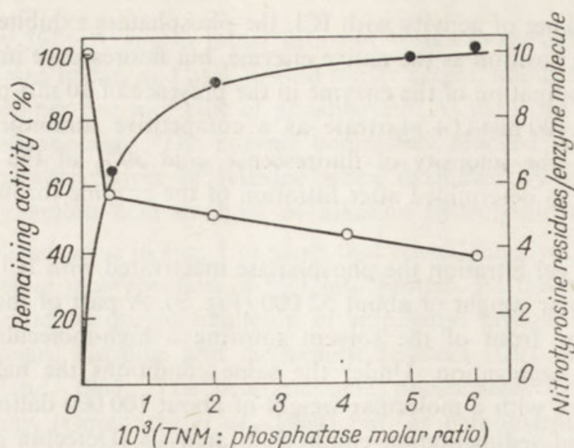
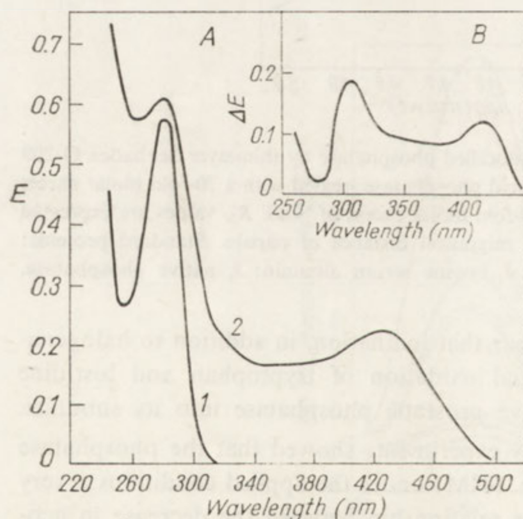



Fig. 4. Effect of molar excess of TNM on the activity of prostatic phosphatase (○), and number of tyrosines modified (●). Conditions as described in Material and Methods.

Spectrophotometric titration of the phosphatase nitrated with a 300-fold molar excess of TNM revealed a shift of the p*K* value from 10.8 for the native enzyme to 7.15 for the modified one (Fig. 6). This value is only slightly higher than the p*K* 6.9 of free 3-nitrotyrosine (Jeckel *et al.*, 1971) which indicates that mainly 3-nitrotyrosyls were formed during nitration of the phosphatase.



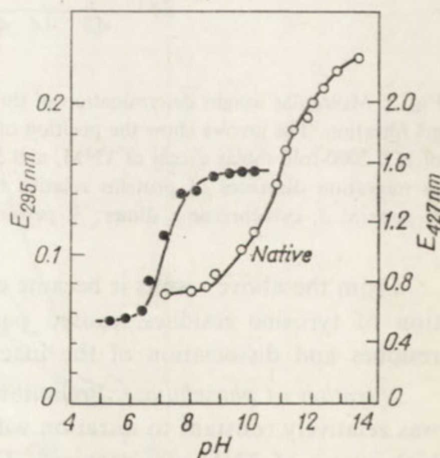




Fig. 5 Fig. 6

Fig. 5. *A*: Absorption spectra of acid phosphatase: *1*, native and *2*, nitrated at a 1300-fold molar excess of TNM. Samples contained about 250 µg of enzyme protein in 1 ml of 0.05 M-Tris-HCl buffer, pH 7.0. *B*: Difference spectrum of the nitrated phosphatase.

Fig. 6. Spectrophotometric titration of (○), native prostatic phosphatase, $E_{295nm}$, and (●), the enzyme nitrated with a 300-fold molar excess of TNM, $E_{427nm}$. The titration was carried out as described by Bobrzecka *et al.* (1968).

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Measurement of extinction at 427 nm determines the amount of nitrotyrosyl residues in protein, provided another yellow reaction product, nitroformate ion, is removed by dialysis or filtration on Dextran gel. It can be seen in Fig. 4 that at an about 300-fold molar excess of TNM and 40% drop in enzymic activity, 6.5 tyrosyl residues of protein underwent nitration. At about 5000-fold molar excess of TNM only 3.5 tyrosyl residues more were nitrated, and the total drop in enzyme activity amounted to 60%.

The maximally nitrated phosphatase retaining 40% of its original activity, was then reduced with dithionite as described by Sokolovsky *et al.* (1967). The nitrated enzyme, 0.2 mg in 1 ml, was incubated for 30 min at 20°C in 0.1 M-Tris-HCl buffer of pH 8.0 containing 1 mM-sodium dithionite. After removal by dialysis of the excess of the reductor, the amount of nitrotyrosyl residues and activity were determined. From the decrease in the extinction at 427 nm it was calculated that all the nitrotyrosyl residues had beed reduced to 3-aminotyrosyls, whereas the activity was only slightly increased, by about 10%, as compared with the activity of nitrated enzyme.

Similarly to the iodinated enzyme, fluorescence intensity of nitrated phosphatase was diminished to about 18% of its original value (Fig. 2). The enzyme treated with TNM in the presence of pNPP showed only a 50% decrease in fluorescence intensity. The same effect was observed in the presence of 5′-AMP or tartrate. Nitrated phosphatase dissociated into subunits with molecular weight of about 55 000 (Fig. 3); at the same time aggregation of the modified phosphatase was observed.

*N-Bromosuccinimide oxidation of phosphatase.* The changes in the absorption spectrum of acid phosphatase titrated with NBS solution at pH 4.5, are presented in Fig. 7. With rising concentration of the reagent, absorption at 280 nm dropped

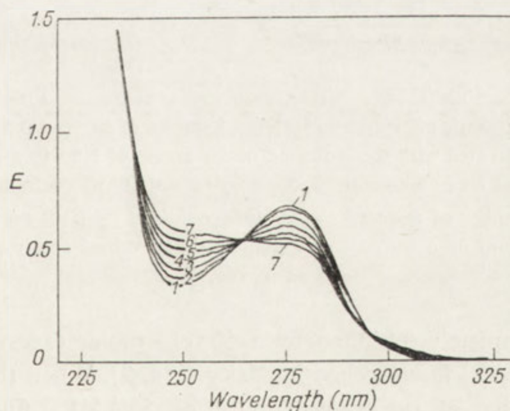


Fig. 7. Changes in u.v. spectra of acid phosphatase associated with the oxidation of the enzyme with NBS. To the enzyme solution, 0.5 mg/ml of 0.1 M-acetate buffer, pH 4.5, an appropriate amount of 3 mM-NBS in the same buffer was added (total volume 2 ml) and after incubation for 3 min at room temp. the absorption spectra were recorded in cuvettes with a 1-cm light path against blanks from which enzyme was omitted. *1*, Native phosphatase; *2 - 7*, phosphatase oxidized with a 10- to 50-fold molar excess of NBS.

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proportionally, while absorption at 250 and 310 nm increased. This type of changes in the protein spectrum suggests participation of tryptophan in the reaction with NBS. Spande & Witkop (1967) demonstrated that, under the influence of NBS, tryptophan groups in protein are oxidized to oxyindole causing changes in the spectrum, mainly in the 280 nm band, which makes possible quantitative analysis of the course of the reaction (Patchornik *et al.*, 1958).

The reaction of acid phosphatase with NBS was immediate and irreversible. Enzyme activity was completely inhibited at a 50-fold molar excess of NBS in the pH range 4.0 - 5.0 (Fig. 8). Dialysis of the enzyme treated with NBS, or Sephadex G-25 filtration, did not restore the activity. When the remaining activity was plotted *versus* the number of tryptophan residues oxidized by NBS (Fig. 9), the decrease in activity was linear and proportional to the number of oxidized tryptophans (up to the seventh one), oxidation of one molecule of tryptophan corresponding to a 10% change in enzyme activity. Oxidation of the eighth tryptophan residue resulted in complete inactivation of the phosphatase.

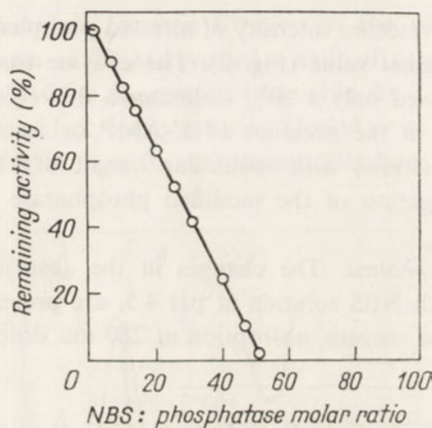


Fig. 8

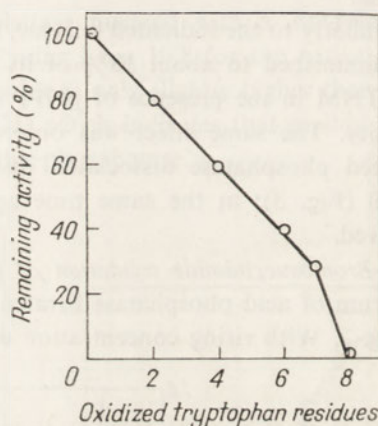


Fig. 9

Fig. 8. Inactivation of prostatic phosphatase by NBS. Samples of enzyme, 0.5 mg/ml of 0.1 M-acetate buffer, pH 4.5, were incubated with the indicated molar excess of NBS in a total volume of 0.5 ml. After 3 min incubation at room temperature, the enzyme activity was determined in 10-μl portions.

Fig. 9. Effect of the number of tryptophan residues oxidized by NBS on the prostatic phosphatase activity. The amount of oxidized tryptophan was determined spectrophotometrically at 280 nm as in Fig. 7.

The enzyme completely inhibited by a 50-fold molar excess of NBS exhibited only 12% of its original fluorescence intensity (Fig. 2). When the enzyme was oxidized in the presence of substrates (pNPP or 5′-AMP), fluorescence intensity amounted to 45 and 67%, respectively, in relation to that of the native enzyme; after removal of NBS by dialysis, 60 and 30% of the original activity was restored.

The phosphatase oxidized with NBS migrated on the thin layer of Sephadex G-200 as a single band with a molecular weight of about 55 000 (Fig. 3); a small amount of the aggregation product was also found.

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*Photochemical oxidation.* When exposed to visible light in the presence of Rose Bengal, the enzyme was readily inactivated. The rate of inactivation was first order and linear on the semi-logarithmic scale down to zero activity after about 30 min illumination at pH 7.4 (Fig. 10). If the enzyme was kept in the dark, there was no loss of activity even in the presence of the sensitizer. The rate of inactivation was markedly dependent upon pH (Fig. 11). A typical sigmoidal curve was obtained with the inflection point near pH 6.0. There appears to be a good agreement between the relative rates of inactivation of the enzyme and the theoretical ionization curve of imidazole group (Westhead, 1965; Freude, 1968). Over the pH range 4.8 - 5.9 the rate of inactivation was low and practically unchanged; beginning with the latter pH value, which is close to p$K$ 5.99 of imidazole ion, the rate of photoinactivation increased rapidly (Fig. 12).

The rate of photoinactivation of the enzyme was markedly reduced in the presence of substrates or competitive inhibitors (Fig. 10 and Table 1). Phosphoethanolamine was the most potent compound: it reduced the rate of photoinactivation at pH 7.0 from 0.163 to 0.026 min$^{-1}$; 3′-UMP showed almost no protective activity, which at present is difficult to explain. Preliminary results of the amino acid analysis of the photooxidized phosphatase indicated that histidine was probably

Table 1

*Influence of substrates and competitive inhibitors on the photoinactivation rate of prostatic phosphatase*

The reaction mixture containing in 1 ml of 0.1 M-Tris-HCl-EDTA buffer, pH 7.0 or 7.4, 50 μg prostatic phosphatase, substrate or inhibitor at indicated concentration, was incubated for 3 min at 20°C in 2-ml spectrophotometric cell. Then 10 μl of Rose Bengal solution (10 mg/ml of buffer) was added, thoroughly mixed and the sample was illuminated from a fixed distance (30 cm) at the same temp. using 600-W projection lamp. Samples, 2 μl of the solution, were withdrawn at different time intervals for measurement of phosphatase activity. The rate constants per minute for the inactivation were calculated after plotting the logarithm of the residual activity against time.

| Compound added | Concentration (mM) | pH | Rate constant for photoinactivation | | Reduction of inactivation rate (%) |
|---|---|---|---|---|---|
| | | | control | with substrate or inhibitor | |
| Phenolphosphate | 45 | 7.0 | 0.163 | 0.129 | 21 |
| β-Glycerophosphate | 180 | 7.0 | 0.163 | 0.126 | 23 |
| L(+)-Tartrate | 10 | 7.4 | 0.193 | 0.096 | 50 |
| Phosphoethanolamine | 45 | 7.0 | 0.163 | 0.026 | 84 |
| Phosphoethanolamine | 45 | 7.4 | 0.193 | 0.033 | 83 |
| Phosphocreatine | 45 | 7.0 | 0.163 | 0.139 | 15 |
| 3′-UMP | 45 | 7.0 | 0.163 | 0.154 | 5 |

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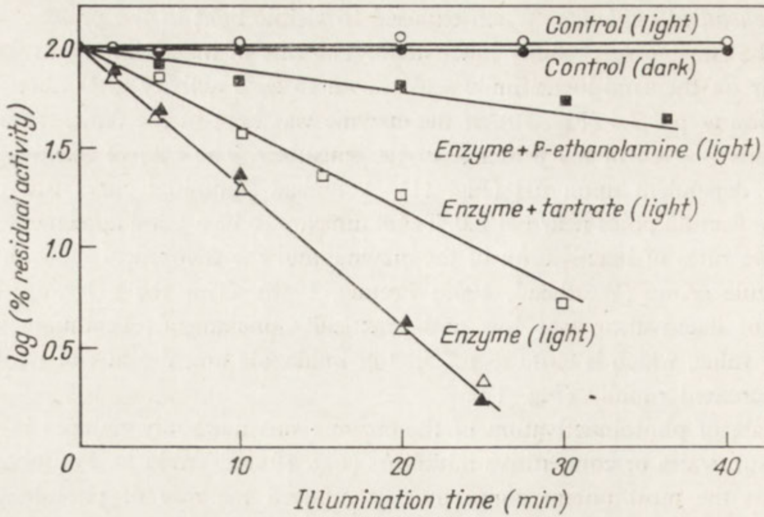


Fig. 10. Photochemical inactivation of prostatic phosphatase at pH 7.4 in the presence of Rose Bengal. Control (light) was the enzyme illuminated without sensitizer; control (dark) was the complete reaction mixture, but not illuminated; △, ▲, experiments carried out with two different enzyme preparations. For details see Methods.

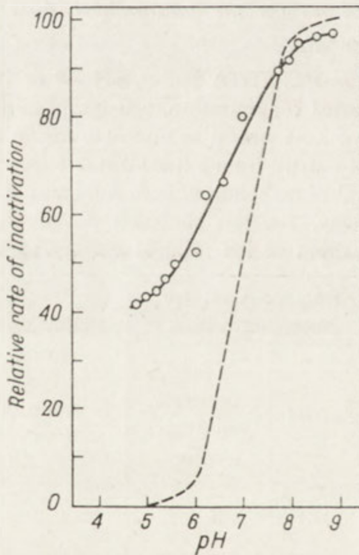


Fig. 11

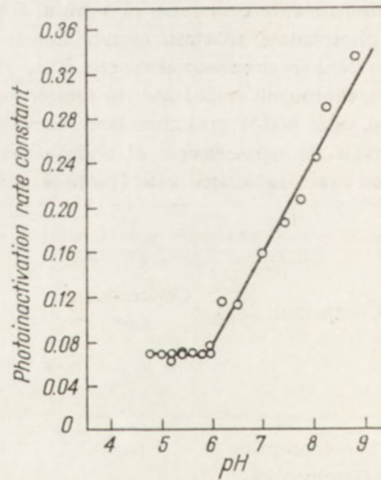


Fig. 12

Fig. 11. Relative rate of prostatic acid phosphatase photoinactivation as a function of pH. Buffers were 0.1 M-sodium acetate (pH 4.8 - 6.5) and Tris-acetate (pH 6.5 - 9.0), containing 1 mM-EDTA. Illumination time was 10 min at 20°C. The dashed line represents the theoretical ionization curve of imidazole.

Fig. 12. Effect of pH on photoinactivation rate constant of prostatic phosphatase. The rate constants per 1 min were calculated from the data obtained in the experiment presented in Fig. 11.

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the only amino acid that was modified. Of the 26 histidine residues present in the native enzyme (Derechin *et al.*, 1971), in the completely photoinactivated enzyme in two experiments 13 and 15 residues were destroyed.

By thin-layer chromatography it was found that, in contrast to the phosphatase iodinated, nitrated, or oxidized with NBS, the photoinactivated enzyme did not undergo dissociation into subunits, and showed but a small tendency to aggregation.

## DISCUSSION

Acid phosphatase from human prostate, unlike many other proteins, shows very low reactivity with iodine, NBS and TNM. Complete blocking of the enzyme activity was attained at a large molar excess of iodine or NBS, whereas an even 6000-fold molar excess of TNM resulted in only 60% inhibition of the enzyme activity. The action of these reagents consists in modifying tyrosine, tryptophan and histidine residues, and moreover they cause dissociation of the enzyme molecule into subunits of mol.wt. about 52 000.

From the results of our present and previous work (Bobrzecka *et al.*, 1968; Rybarska, 1970) it follows that iodination resulting in complete inhibition of the enzyme activity modifies 13 tyrosine residues out of 43 present in the enzyme molecule. Spectrophotometric titration and treatment of the phosphatase with tyrosinase demonstrated that most of the tyrosine residues were buried within the polypeptide chain. Nitration of the phosphatase with TNM resulted in formation of about 10 nitrotyrosine residues with simultaneous loss of about 60% of the enzyme activity.

These results suggest that tyrosine is not involved in the catalytic centre of the enzyme but probably plays a role in stabilization of its active conformation.

Iodination acts also on tryptophan residues, modifying 1.3 out of 18 present in a molecule of the acid phosphatase, and causing an about 80% decrease in fluorescence intensity of the emission spectrum at the maximum characteristic for exposed tryptophan residues (Chen *et al.*, 1969). A similar decrease in fluorescence intensity was observed under the influence of TNM, which indicates that this reagent also had a modifying effect on tryptophan residues (Sokolovsky *et al.*, 1970).

Oxidation by NBS, which in other proteins acts most readily on SH groups (Cohen, 1968), had no effect on SH groups of the phosphatase (Bobrzecka *et al.*, 1968), whereas 8 out of 18 tryptophan residues present in the enzyme molecule, underwent oxidation. The decrease in activity was proportional to the number of oxidized tryptophan residues. Increasing of the concentration of NBS did not further increase the number of oxidized tryptophans, indicating that only the exposed tryptophan residues were oxidized, other being inaccessible under the experimental conditions used.

Gradual oxidation of the phosphatase with NBS caused a progressive drop in the intensity of fluorescence of the enzyme protein; after complete blocking of biological activity, the fluorescence decreased to about 10% of its original intensity.

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In the experiments in which prostatic phosphatase was modified in the presence of substrates or tartrate as a competitive inhibitor, the loss of activity due to iodination, nitration or oxidation with NBS was markedly lower. This indicates that tyrosine and especially tryptophan residues may be involved in creation of a specific apolar environment (Nigam *et al.*, 1959) in which the enzyme-substrate complex can be formed (London *et al.*, 1958) but do not participate directly in the catalytic reaction.

In view of the possible role of histidine residues in catalytic activity of prostatic phosphatase (Ostrowski & Barnard, 1973), photochemical studies were undertaken. In contrast to the severity of conditions under which the enzyme reacted with ICl, TNM or NBS, photooxidation in the presence of Rose Bengal proceeded readily; however, the modification of the protein molecule did not lead to dissociation into subunits. The pH profile for photoinactivation of prostatic phosphatase (cf. Fig. 11) is quite compatible with the theoretical ionization curve for imidazole group (Westhead, 1965). A similar pH-dependent photooxidation of several enzymes containing histidine residues in the active centre has been reported (Westhead, 1965; Takahashi & Seifert, 1969; Fishman *et al.*, 1973). As shown by Fishman *et al.* (1973), in addition to histidine, also tryptophan, methionine, tyrosine and cysteine are photosensitive in varying degrees, but the pH-dependence of photooxidation of these amino acids is different as compared with that of histidine. The influence of substrates and competitive inhibitor on photoinactivation of prostatic phosphatase (cf. Table 1) suggests that the loss of its activity is connected with photodynamic events at, or near, the active site of the enzyme.

The above observations are in good agreement with the results on phosphoryl-enzyme intermediate in the catalytic reaction of the acid phosphatase from human prostate (Ostrowski & Barnard, 1971, 1973). On the basis of the transphosphorylation reaction, inhibition with DFP and titration of active sites, it was concluded that the mechanism of enzyme activity consists in formation of a catalytic covalent E-P intermediate. This intermediate is stable at alkaline pH and unstable at acidic pH, which points to the presence of histidine in the catalytic site of the acid phosphatase from prostate. Similar experimental data on the structure of active site have been obtained with acid phosphatases isolated from rat liver (Igarashi *et al.*, 1970) and wheat germs (Hickey & VanEtten, 1972).

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## KWAŚNA FOSFATAZA STERCZU LUDZKIEGO
### MODYFIKACJA CHEMICZNA I AKTYWNOŚĆ ENZYMU

### Streszczenie

Chlorek jodu (JCl) przy 70-krotnym molowym nadmiarze i *N*-bromobursztynyloimid (NBS) przy 50-krotnym nadmiarze całkowicie hamują aktywność fosfatazy sterczu ludzkiego; tetranitrometan (TNM) przy 300-krotnym molowym nadmiarze hamuje enzym tylko w 40%. JCl i TNM reagują głównie z resztami tyrozynowymi, natomiast NBS utlenia głównie tryptofan. Modyfikowany za pomocą powyższych odczynników enzym ulega równocześnie dysocjacji na podjednostki o masie 52 000 - 55 000 daltonów. Intensywność fluorescencji zmodyfikowanego enzymu ulega znacznemu

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obniżeniu w paśmie widma emisyjnego charakterystycznym dla eksponowanych do solwentu reszt tryptofanowych (355 nm).

Fosfataza ulega stosunkowo szybkiej fotoinaktywacji w obecności Różu Bengalskiego jako barwnika uczulającego. Szybkość inaktywacji jest pierwszego rzędu i ulega znacznemu obniżeniu w obecności substratów lub kompetytywnego inhibitora. Szybkość fotoinaktywacji jest stała w zakresie pH 4.8 - 6.0 i szybko wzrasta przy wyższych wartościach pH, przebiegając równolegle z teoretyczną krzywą jonizacji imidazolu. Fotoinaktywacja prowadzi do modyfikacji ok. 14 reszt histydynowych enzymu na 26 obecnych, ale nie powoduje dysocjacji fosfatazy na podjednostki.

Analiza wyników wskazuje, że prawdopodobnie reszty histydynowe biorą bezpośredni udział w katalitycznej funkcji enzymu, podczas gdy reszty tyrozynowe i tryptofanowe nie są zaangażowane bezpośrednio w tym procesie.

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## RECENZJE KSIĄŻEK

TECHNIQUES IN PROTEIN BIOSYNTHESIS, vol. 3. P. N. Campbell and J. R. Sargent, eds. Academic Press, London - New York, 1973. str. 1 - 265, cena £ 5.90.

Książka należy do serii wydawniczej, zapoczątkowanej w 1964 r., dotyczącej metod w badaniu biosyntezy białka. Chociaż wiadomości na ten temat gwałtownie rozwijają się i zmieniają, to jednak opisane techniki są w większości trwałe i powszechnię stosowane.

Omawiany tom 3 zawiera pięć rozdziałów. Pierwszy z nich, opracowany przez M. Cannona, jest zatytułowany: „Zastosowanie antybiotyków i innych inhibitorów do badania biosyntezy białka w układach bakteryjnych". Zawiera on krótki opis mechanizmu biosyntezy białka oraz szczegółowe dane dotyczące działania antybiotyków na poszczególne etapy syntezy peptydów. Przedstawiono metody badania funkcji rybosomów w procesie inicjacji i elongacji oraz terminacji syntezy peptydów. Wykazano również rolę różnych inhibitorów w wyświetlaniu tych mechanizmów.

Rozdział drugi, opracowany przez R. W. Turkingtona, dotyczy warunków hodowli komórek zwierzęcych i metod badania biosyntezy białka i kwasów nukleinowych. Przykładem są hodowle komórek gruczołu mlecznego myszy. Układy tego typu są szczególnie dogodne do badania problemów różnicowania się komórek i mechanizmów regulacji syntezy makrocząsteczek, a także ich transportu międzykomórkowego. Przedstawiono różne techniki hodowli komórek i zapotrzebowanie składników do ich rozwoju, jak soli, aminokwasów, źródeł energii, witamin, hormonów.

Rozdział trzeci, opracowany przez J. C. Turnera, jest krytycznym przeglądem oznaczania radioaktywności za pomocą technik scyntylacji w płynach. Zawiera on historyczny i teoretyczny rys używanych metod z propozycjami zastosowania ich do określonych materiałów i sytuacji. Poruszony jest problem rozpuszczalników, podłoża stałego, gazów i par, wydajności liczenia i gaszenia, scyntylatorów, techniki podwójnego znakowania oraz zastosowania komputera do przeliczeń.

Czwarty rozdział, napisany przez M. A. Williamsa, dotyczy zastosowania autoradiografii do badań nad biosyntezą białka w mikroskopie elektronowym. W rozdziale tym przedstawiono zasady i znaczenie tej metody w wyjaśnianiu struktury komórki i lokalizacji zachodzących w niej procesów. Wymieniono używane do tego celu izotopy i opisano metody przygotowania skrawków oraz całą procedurę dotyczącą obserwacji w mikroskopie elektronowym łącznie z oceną wyników.

Rozdział ostatni, opracowany przez H. Glaumanna, zawiera opis metod stosowanych do frakcjonowania błon wewnątrzkomórkowych ze szczególnym uwzględnieniem syntezy i transportu białek. Na przykładzie głównie komórek wątroby przedstawiono techniki frakcjonowanego wirowania organelli oraz składników błon komórkowych. Omówiono skład chemiczny tych frakcji, a także strukturę rybosomów i poglądy dotyczące mechanizmów biogenezy błon wewnątrzkomórkowych.

Książka stanowi bardzo wartościową pozycję i jest szczególnie użyteczna dla tych, którzy rozpoczynają badania w dziedzinie biosyntezy białka, a także dla tych, którzy pragną poszerzyć w niej swoje wiadomości.

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ADVANCES WITH ZONAL ROTORS. E. Reid, ed. Longman Group Ltd, London 1973; str. XI+275; cena £ 3.—.

„Advances with Zonal Rotors" jest trzecim tomem z serii „Methodological Developments in Biochemistry", wydawanej przez Longman Group. Zadaniem tej serii jest dostarczenie w możliwie prostej formie zespołu informacji metodycznych związanych z określonymi technikami biochemicznymi. Książka jest dziełem zbiorowym, opartym na materiałach przedstawionych na sympozjum poświęconym wirowaniu przy zastosowaniu rotorów zonalnych. Sympozjum to zorganizowane było w 1971 r. przez Wolfson Bioanalytical Center (University of Surrey).

Przedstawiony materiał zgrupowany jest w kilku częściach. Jako wprowadzenie służą artykuły D. T. Prospero, G. D. Birnie i J. A. T. P. Meuwissena poświęcone teorii sedymentacji makromolekuł w gradiencie gęstości, ze szczególnym uwzględnieniem wirowania przy użyciu rotorów zonalnych. W artykułach tych rozważane są czynniki wpływające na optymalne rozdzielenie badanych substancji. Omówione są też czynniki decydujące o pojemności gradientu (gradient capacity) oraz zastosowania wirowania równowagowego i sedymentacyjnego — dwóch podstawowych sposobów rozdziału przy wirowaniu w gradiencie stężeń.

Część następną stanowią artykuły poświęcone problemom technicznym, jak na przykład konstrukcji przepływowego rotoru zonalnego, znajdującego zastosowanie przemysłowe przy otrzymywaniu cząstek wirusowych z kultur tkankowych i produkcji szczepionek przeciwwirusowych.

Dalsza część książki poświęcona jest rozważaniom nad szczegółowymi przykładami rozdzielania różnych substancji przy użyciu rotorów zonalnych. Przedstawione tu zostały przykłady rozdziału: białek, ze szczególnym uwzględnieniem lipoproteidów; kwasów nukleinowych i rybonukleoproteidów, organelli komórkowych i wreszcie różnych typów komórek.

Zastosowanie wirowania w rotorze zonalnym do rozdziału mieszanin białkowych pozwala oznaczyć stałe sedymentacji badanych białek i jednocześnie wydzielić je z mieszaniny w znaczących ilościach. Może najciekawszym przykładem zastosowania zonalnego rotoru do rozdzielania mieszanin białkowych jest frakcjonowanie plazmy krwi ludzkiej metodą opracowaną przez G. B. Clyne i współpracowników. Poprzez zastosowanie wirowania w gradiencie stężeń sacharozy w rotorze zonalnym, otrzymali oni z plazmy krwi wysoko oczyszczonej czynnik VIII, ułatwiający krzepnięcie krwi u osób chorych na hemofilię typu A. Zastosowana procedura może służyć do produkcji tego białka w dużych ilościach. Stwarza to poważne możliwości w leczeniu zachowawczym hemofilii.

Doskonały artykuł R. A. Coxa i H. Pratta poświęcony jest otrzymywaniu rybosomów i podjednostek rybosomowych. Zostały tu przejrzyście zestawione warunki wirowań umożliwiające otrzymanie aktywnych biologicznie podjednostek rybosomowych z rybosomów wydzielonych z organizmów eukariotycznych.

Wreszcie w ostatniej części książki zwraca uwagę metoda opracowana przez G. Sieberta i współpracowników, poświęcona wirowaniu w środowisku niewodnym. Stosując wirowanie zonalne w gradientach otrzymanych poprzez zmieszanie cykloheksanu z czterochlorkiem węgla, autorzy ci wydzielili wysoko-oczyszczoną frakcję jąder komórkowych.

Omawiana książka dostarcza informacji niezbędnych każdemu, kto w praktyce laboratoryjnej styka się z zastosowaniem wirowań w gradiencie gęstości i powinna być ze szczególną uwagą przeczytana przed przystąpieniem do wirowań przy użyciu rotora zonalnego.

*Włodzimierz Zagórski*

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