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W. OSTROWSKI, Z. WALCZAK*, L. STASIUK and M. LASKOWSKI, Sr.**

PROPERTIES AND SPECIFICITY OF RIBONUCLEASE IIA FROM *THIOBACILLUS THIOPARUS*

Interfaculty Department of Physiological Chemistry, Medical School, ul. Kopernika 7, Kraków, Poland
The Laboratory of Enzymology, Roswell Park Memorial Institute, Buffalo, New York 14203, U.S.A.

1. RNase IIA from *Thiobacillus thioparus* is a non-cyclizing endonuclease with an activity optimum at pH 7.0. It withstands elevated temperatures and low pH. The molecular weight determined by sucrose gradient centrifugation is 13 500. 2. With yeast RNA as substrate RNase IIA produces all four mononucleotides (8% of the products), di- and trinucleotides and an undialysable "core". All products are terminated with a 3′-monophosphoryl group. The "core" contains 60% guanine. 3. Purines predominate at the 5′-terminus of oligonucleotides and only pyrimidines occur at the 3′-terminus. 4. It is concluded from the above that RNase IIA from *Th. thioparus* is similar to, but not identical with, pancreatic RNase.

Isolation from *Thiobacillus thioparus* cells of three ribonucleases differing in pH optimum and the rate of thermal denaturation, has been described in our previous communications (Ostrowski & Walczak, 1961, 1963; Walczak & Ostrowski, 1964). All three enzymes, with pH optima at 5.0, 7.0 and 9.5, respectively, which were separated from each other by column chromatography on CM-, DEAE-cellulose and Amberlite IRC-50, hydrolysed yeast RNA leaving a "core" which differed in base composition from that resulting from the action of pancreatic RNase (Walczak & Ostrowski, 1964). The enzyme with pH optimum at 7.0, called RNase IIA, which accounts for about 60% of the total nucleolytic activity of the cells, produced, when acting on yeast RNA, a "core" with a much higher content of guanine than of adenine. This observation warranted an additional examination of specificity of the enzyme.

In the present work, further properties of RNase IIA from *Th. thioparus* are described and some data on its specificity presented. The results obtained indicate

* Present address: Chemical Laboratory of the III Surgical Clinic of the Medical School in Kraków, Poland.

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that RNase IIA is an endonuclease producing fragments with terminal phosphate groups in 3′-position. The enzyme possesses specificity similar to, although not identical with, that of pancreatic ribonuclease.

MATERIALS AND METHODS

The conditions of *Thiobacillus thioparus* culture, harvesting, and removal of elemental sulphur were as described previously (Ostrowski & Krawczyk, 1957; Ostrowski & Walczak, 1961).

Yeast RNA, prepared by the method of Crestfield, Smith & Allen (1955), was purchased from Worthington Biochem. Corp. (Freehold, N.J., U.S.A.) and additionally purified in the following way. Aqueous solution of RNA was dialysed against water at 4° for 24 hr., then 2 vol. of ethanol containing 1% sodium acetate was added, and the collected RNA sediment was washed with ethanol, ether, and dried *in vacuo*.

Highly polymerized DNA from calf thymus was prepared by the method of Zamenhof, Griboff & Marullo (1954).

Exonuclease from *Crotalus adamanteus* venom was prepared according to Richards, Tutas, Wechter & Laskowski (1967), and 5′-nucleotidase after Sulkowski, Björk & Laskowski (1962). Highly purified phosphomonoesterase from human prostate was obtained by the procedure described by Ostrowski & Tsugita (1961), in the modification of Ostrowski (1968).

CM- and DEAE-cellulose, of 0.66 and 0.60 mEq/g. capacity, respectively, were purchased from Serva Co. (Heidelberg, West Germany); Amberlite IRC-50 (XE 64), 200-400 mesh, from Koch-Light (Colnbrook, Bucks., England); and Amberlite MB3 resin, from Mallinckrodt Chemical Co. (St. Louis, Mo., U.S.A.).

Determination of ribonuclease. The activity was assayed using yeast RNA by the method of Anfinsen, Redfield, Choate, Page & Carroll (1954) in the modification described previously (Ostrowski & Walczak, 1961). The enzyme activity was expressed in E_{260} units; one unit being defined as that amount of enzyme which under the standard conditions caused an increase of extinction of the acid-soluble products equal to 1.0.

Determination of other enzymes. Deoxyribonuclease was assayed after Kunitz (1950) with thymus DNA as substrate. Phosphomonoesterase and phosphodiesterase were assayed using as substrates *p*-nitrophenylphosphate and bis-*p*-nitrophenylphosphate (Sigma Chem. Corp., St. Louis, Mo., U.S.A.), respectively, according to the procedure described previously (Ostrowski, 1961).

Preparation of RNase IIA. The procedure is a modification of the method described in earlier communications (Ostrowski & Walczak, 1961; Walczak & Ostrowski, 1964). The *Th. thioparus* cell paste was ground for a few hours with carborundum powder in a porcelain mortar in a cold room, then suspended in 0.25 N-H_2SO_4 and left overnight in the cold room. The suspension was adjusted to pH 6.8 with NaOH, the sediment was centrifuged off at 12 000 *g*, and to the supernatant

solid ammonium sulphate was added to attain 0.55 saturation. The precipitate was centrifuged off at 6000 *g*, and to the supernatant ammonium sulphate was added to 0.85 saturation. After 24 hr. in the cold room, the precipitate was collected by centrifugation at 15 000 *g*, dissolved in a small amount of 0.01 M-Na-phosphate buffer, pH 6.0, dialysed against the same buffer, and adsorbed on a CM-cellulose column (20×3.5 cm.) equilibrated with the above buffer solution. The column was washed and RNase I eluted with 800 ml. of the starting buffer, then RNase II was eluted with a convex pH gradient obtained with 0.01 M-Na-phosphate buffer, pH 6.0, and 0.15 M-Na-phosphate buffer, pH 7.5. A typical elution pattern is presented in Fig. 1. Fractions nos. 50 - 60 were pooled, dialysed against water and freeze-dried. About 50 mg. of the preparation was dissolved in 2 ml. of 0.2 M-Na-phosphate buffer, pH 6.5, and adsorbed on an Amberlite IRC-50 column (25×1 cm.) equilibrated with the same buffer. The elution with 250 ml. of the buffer gave two active peaks: RNase IIA and RNase IIB (Fig. 2). The fractions containing RNase IIA were pooled, dialysed against water and freeze-dried.

This preparation of RNase IIA had an activity of about 130 E_{260} units/mg. of protein, and was used in experiments described below. The enzyme was purified about 350-fold with respect to the activity of the crude extract. The yield in relation to the total nucleolytic activity was 5%. The preparation exhibited low phosphomonoesterase activity and was free of DNase and non-specific phosphodiesterase, although the latter enzyme had also been found in *Th. thioparus* cells (Ostrowski, 1961).

Preparation of yeast RNA hydrolysates obtained by digestion with RNase IIA. Hydrolysate I. To 75 mg. of RNA dissolved in 10 ml. of water and adjusted to pH 7.0 with NH_3, 2 mg. of RNase IIA was added and the mixture was incubated for 24 hr. at 37°, a few drops of ammonia being added from time to time to maintain the pH at about 7.0. After incubation, the solution was freeze-dried and kept until required.

Hydrolysate II. To samples of yeast RNA at concentrations corresponding to $E_{260}^{1cm} = 100$ in 3.75 ml. of 0.05 M-tris-HCl buffer, pH 7.0, was added 100 µl. of aqueous solution of RNase IIA (5 mg./ml.). After incubation at 37° for 15, 60 or 180 min., the mixture was adjusted to pH 5.0 and used for analyses described below.

Determination of terminal nucleotides. The solution of oligonucleotides (5-10 mg. in 2 ml. of water) obtained by exhaustive hydrolysis of yeast RNA with RNase IIA (hydrolysate I) was adjusted to pH 5.0, 20 µl. of prostate phosphomonoesterase solution (about 1.5 mg. of protein/ml.) was added, and the mixture was incubated at 37° for 1 hr.; then another 20 µl. of the phosphomonoesterase solution was added and the incubation continued for the additional 2 hr. The mixture was adjusted to pH 7.0 with a KOH solution and heated at 60° for 2 min. to inactivate the enzyme. The sample was diluted twofold with 2 N-KOH, incubated at 18° for 20 hr., and submitted to chromatography on Whatman 3 MM paper in *n*-propanol - conc. NH_3 - water (55:10:35, by vol.) solvent. The nucleosides and nucleotides were located under UV light, eluted with water, hydrolysed with 60% $HClO_4$ at

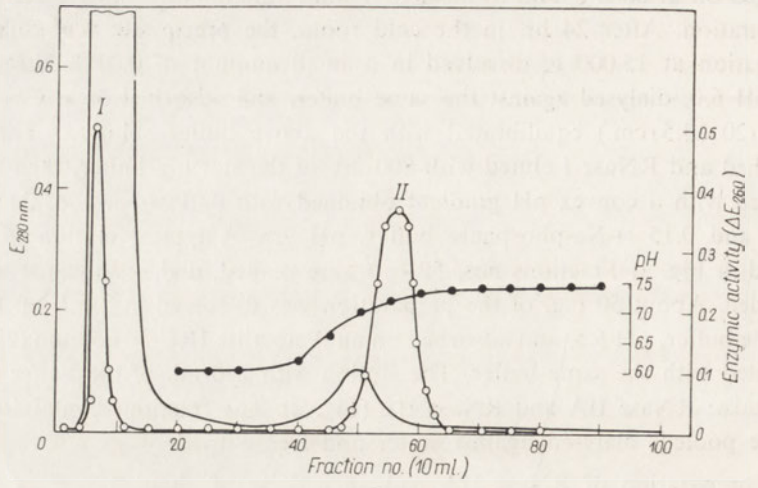

Fig. 1. Separation of RNase I from RNase II by CM-cellulose column chromatography. Conditions of elution: the mixing chamber contained 250 ml. of 0.01 M-Na-phosphate buffer, pH 6.0, the reservoir 0.15 M-Na-phosphate buffer, pH 7.5. Fractions of 10 ml. were collected at a rate of 100 ml./hr. (———), E_{280}; (○), enzyme activity; (●), pH gradient.

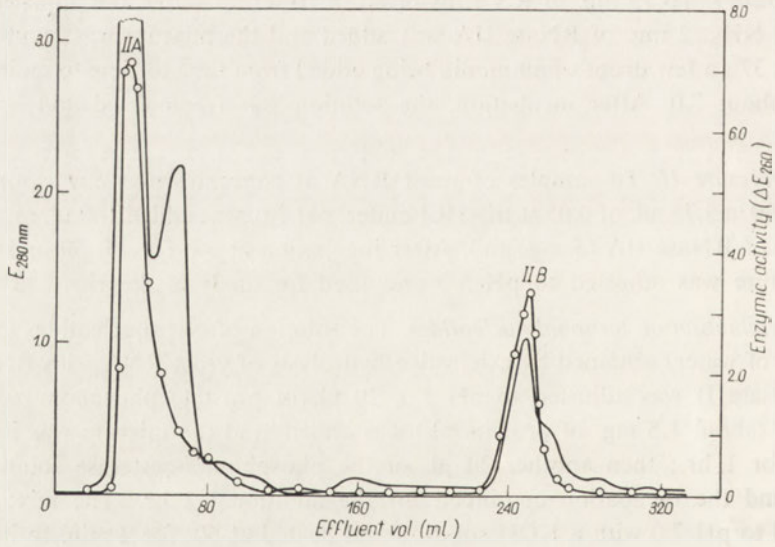

Fig. 2. Separation of RNase IIA from RNase IIB by Amberlite IRC-50 column chromatography. The pooled fractions nos. 50 - 60 from CM-cellulose (see Fig. 1) were applied to the column and eluted with 0.2 M-Na-phosphate buffer, pH 6.5. (———), E_{280}; (○), enzyme activity.

100° for 1 hr., and the bases separated by chromatography on Whatman no. 1 paper in the solvent: isopropanol - HCl - water (Wyatt, 1951). The individual bases were eluted with 0.1 N-HCl, and extinction at 257 nm was measured in a Uvispec spectrophotometer (Hilger & Watts, London, England). The concentration of the bases was estimated using the molar extinction coefficients cited by Dawson, Elliott, Elliott & Jones (1959).

To samples of hydrolysate II, adjusted to pH 5, were added 3.5 units of snake venom exonuclease and the mixture incubated at pH 5.0 for 20 hr. at 37°. This procedure is based on the observation that the optimum for digestion of oligonucleotides with a terminal phosphate at 3′-position is at pH 6 (Richards & Laskowski, 1969), and not at pH 9 as assumed previously. The pH 5.0 instead of 6.0 was used because the samples after hydrolysis were immediately subjected to continuous-flow electrophoresis at pH 5.0.

Column chromatography of oligonucleotides. The chromatography on DEAE-cellulose column in ammonium-carbonate buffer, pH 8.6, after Staehelin (1961) was employed. Hydrolysate I, about 75 mg., was dissolved in 2 ml. of 0.01 M-NH_4HCO_3 - NH_3 buffer, pH 8.6, and adsorbed on the column (50×0.9 cm.) equilibrated with the same buffer. After washing the column with 200 ml. of the buffer, the nucleotides were eluted with a concave concentration gradient (from 0.01 M to 0.04 M) of the same buffer. To obtain the gradient, two conical vessels, containing 750 ml. of the starting or eluting buffer, were employed. The course of elution was checked by measuring the extinction at 260 nm. The fractions corresponding to the particular peaks were pooled and freeze-dried.

For determination of the base sequence of the obtained di- and tri-nucleotide fractions, they were submitted to dephosphorylation by prostate phosphomonoesterase, then to the action of snake venom exonuclease and hydrolysis with KOH, according to the procedure described by Burton (1965) and Laskowski (1966).

Fractionation of nucleotides by continuous-flow electrophoresis. This was carried out in a model FF separator (Brinkmann Instruments Inc., Westbury, N.Y., U.S.A.) according to the method described by Sulkowski & Laskowski (1967). In the separation chamber, 0.05 M-ammonium-acetate buffer, pH 5.0, was employed. The same buffer of 0.15 M concentration served for rinsing of the electrodes. The rate of inflow of the sample was 1.5 ml./hr., and the separation was carried out at 1600 V and 180 mA, at 5°.

Sucrose-density-gradient centrifugation. This was carried out in an Omega ultracentrifuge (M. Christ, Osterode, West Germany) in a swinging bucket rotor no. 001. The sucrose gradient, 5 - 20% (w/v), in 0.05 M-tris-HCl buffer, pH 7.0, was prepared after Britten & Roberts (1960), and the results were analysed according to Martin & Ames (1961). The standard proteins used were: ox liver catalase (Koch-Light, Colnbrook, Bucks., England) and crystalline equine HbO_2 prepared according to the method of Heidelberger (1922). Catalase was determined by changes in extinction at 240 nm of the decomposed H_2O_2; HbO_2 was measured at 417 nm.

RESULTS

General properties of RNase IIA. Ribonuclease IIA digested only RNA, whereas it hydrolysed neither bis-*p*-nitrophenylphosphate nor native or denatured DNA (Fig. 3).

The pH optimum of the enzyme was determined in different buffer solutions of 0.075 ionic strength, with yeast RNA as substrate (Fig. 4). The pH-activity curve exhibited a rather sharp peak at pH 7.0, but at pH 5.5 and 10.0 there was still about 50% of the maximum activity.

The effect of ionic strength on RNase IIA activity is shown in Fig. 5. The increasing NaCl concentration in 0.05 M-phosphate buffer of pH 7.0, caused a decrease of enzyme activity, which at 1 M-NaCl concentration was by about 75% lower than when the buffer containing no salt was employed.

The effect of heating was studied at pH 7.0 in 0.05 M-tris-HCl buffer. After 1 hr. at 100° the enzyme lost about 30% of the initial activity (Fig. 6). The temperature optimum with yeast RNA as substrate, was at about 50° (Fig. 7).

The effect of bivalent cations and other factors on RNase IIA activity was studied in 0.1 M-triethylamine-HCl - NaOH buffer, pH 7.0. Some cations had a non-specific

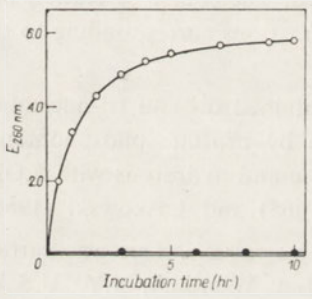

Fig. 3

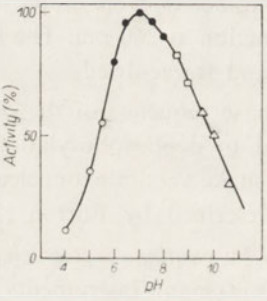

Fig. 4

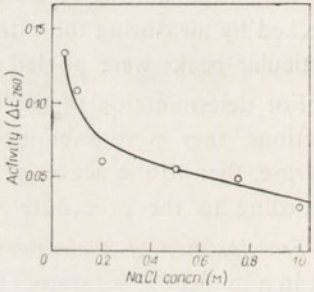

Fig. 5

Fig. 3. The course of hydrolysis of (○), yeast RNA and (●), thymus DNA by RNase IIA. To 5 ml. of 0.6% solution of substrate in 0.1 M-citrate-phosphate buffer, pH 7.0, was added 1 ml. (0.5 mg.) of enzyme solution, and the mixture was incubated at 37°. At the time intervals indicated, 0.2-ml. samples were withdrawn, 0.3 ml. of 2.5% perchloric acid solution containing 0.25% of uranyl acetate was added and, after centrifugation in the cold room, the acid-soluble products were determined at 260 nm. A substrate solution without the enzyme, incubated under the same conditions, served as control.

Fig. 4. The effect of pH on the activity of RNase IIA. The reaction mixture contained: 0.4 ml. of 0.2 M-buffer solution, 0.4 ml. of 0.6% aqueous RNA solution and 0.4 ml. (0.2 mg.) of enzyme solution. After incubation (37°, 60 min.) the amount of acid-soluble products was measured at 260 nm. (○), Acetate; (●), Na-phosphate; (□), tris-HCl; and (△), glycine-NaOH buffer.

Fig. 5. The effect of ionic strength on the activity of RNase IIA. The substrate, 0.6% RNA in 0.05 M-Na-phosphate buffer, pH 7.0, containing varying NaCl concentrations, was incubated with the enzyme at 37° for 60 min.

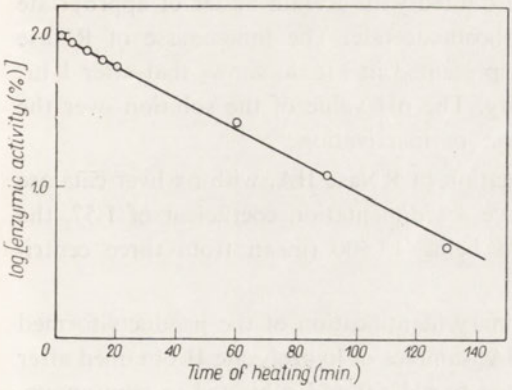

Fig. 6

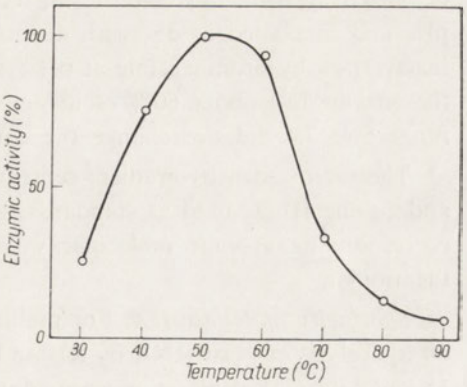

Fig. 7

Fig. 6. Time-course of thermal denaturation of RNase IIA at 100°. The enzyme, 0.1 mg. in 1 ml. of 0.05 M-tris-HCl buffer, pH 7.0, was heated for the time indicated. After cooling, the activity was determined.

Fig. 7. The effect of temperature on the activity of RNase IIA. The substrate, 0.4 ml. of 0.6% RNA, and 0.1 ml. (0.05 mg.) of enzyme in 0.05 M-tris-HCl buffer, pH 7.0, were incubated for 30 min. at the indicated temperature.

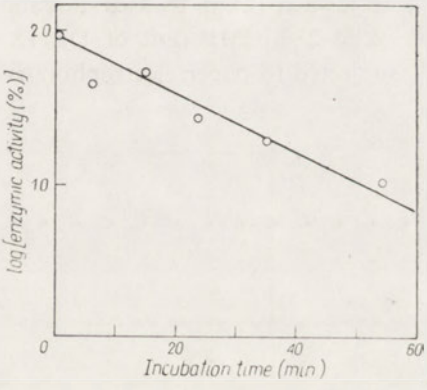

Fig. 8. Time-course of inactivation of RNase IIA by bromoacetate at pH 5.8. The enzyme solution was deionized on Amberlite MB3 column (6×1.2 cm.), then diluted with acetate buffer, pH 5.8, and incubated at 35° with 40 mM-bromoacetate. At indicated time intervals, samples were withdrawn for RNase determination.

effect on the enzyme, detectable at concentrations of 10 mM (after 30 min. incubation at 37°), and accompanied by a visible precipitation (Cd^{2+}, Cu^{2+}, Zn^{2+}), whereas Ca^{2+}, Co^{2+}, Fe^{2+}, and Mg^{2+} had no effect under these conditions. Other reagents at 1mM concentrations either did not influence the enzyme activity at all (EDTA and *p*-chloromercuribenzoate), or their effect did not exceed a few percent. Ascorbic acid, α,α'-dipyridyl and 8-hydroxyquinone enhanced, and cysteine, thioglycollate, glutathione and iodoacetate decreased the activity.

On the other hand bromoacetate, which strongly inhibits pancreatic ribonuclease by carboxymethylation of histydyl residues in position 119 (Glick, Goren & Barnard, 1967), had a marked influence on the activity of RNase IIA. The enzyme was first

deionized by Amberlite MB3 resin, then diluted with acetate buffer of appropriate pH, and incubated at 35° with 40 mM-bromoacetate. The time-course of RNase inactivation by bromoacetate at pH 5.8, presented in Fig. 8, shows that after 1 hr. the enzyme lost about 60% of its activity. The pH value of the solution over the range 5.4 - 7.4 did not change the course of inactivation.

The sucrose-density-gradient centrifugation of RNase IIA, with ox liver catalase and equine HbO_2 used as standards, gave a sedimentation coefficient of 1.57, the corresponding average molecular weight being 13 500 (mean from three centrifugations).

Specificity of RNase IIA. For preliminary identification of the products formed on hydrolysis of yeast RNA by RNase IIA, samples of hydrolysate II obtained after 15 and 180 min. of digestion were adjusted to pH 5.0 and subjected to continuous-flow electrophoresis (Fig. 9). The increase of the peak of nucleosides (tubes nos. 6 - 8) with time of digestion confirms the presence of contaminating phosphomonoesterase. The peak of mononucleotides (tubes nos. 18 - 28) was rather small after 15 min. of digestion and increased after 180 min. As demonstrated by spectral analysis, this peak contained all four mononucleotides. In further fractions (tubes nos. 30 - 46), di-, tri-, tetra- and higher oligonucleotides were present; the amount of oligonucleotides after 180 min. of hydrolysis was markedly decreased. Figure 9 points also to the absence of cyclic mononucleotides.

RNase IIA possessed no ability to hydrolyse cyclic 2′,3′-nucleotides. When cyclic 2′,3′-CMP (gift of Dr. D. Shugar) was incubated with the enzyme and subjected to paper electrophoresis at pH 3.5 (Markham & Smith, 1952), it showed

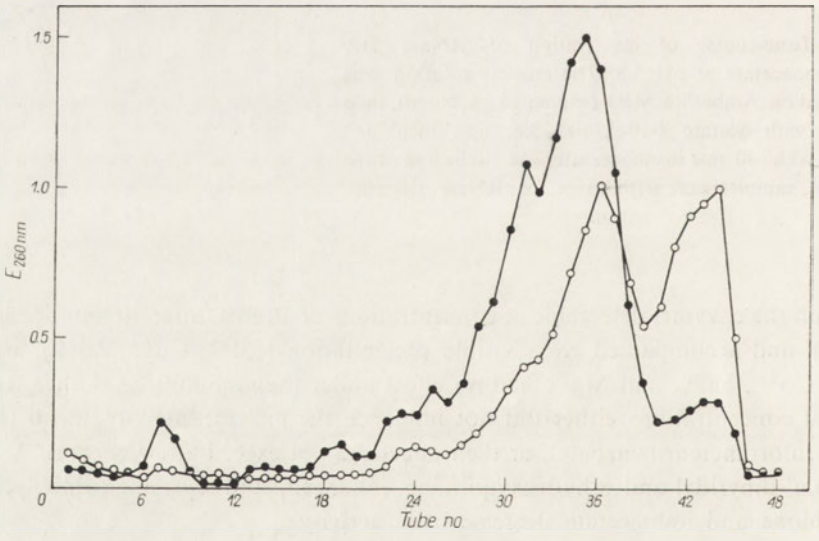

Fig. 9. Fractionation by continuous-flow electrophoresis of the products obtained from yeast RNA by RNase IIA treatment (hydrolysate II). Digestion time: (○), 15 min.; (●), 180 min. For details of electrophoresis see Materials and Methods.

the same mobility as the non-treated sample. Under the same conditions of electrophoresis, the mobility of 3'-CMP was quite different.

Diffusible products of prolonged digestion of RNA. A sample of yeast RNA, 20 mg., and 0.5 mg. of RNase IIA dissolved in 2 ml. of 0.1 M-ammonium-carbonate buffer, pH 7.0, were dialysed at 37° against 10 ml. of the same buffer. At indicated time, the outer fluid was replaced by a fresh buffer, and the removed fluid, after determination of extinction at 260 nm, was freeze-dried. The dry residue was dissolved in a small volume of 70% $HClO_4$, hydrolysed at 100° for 1 hr., and the base composition determined by paper chromatography (Wyatt, 1951).

The results presented in Table 1 show that the rate of RNA hydrolysis expressed as E_{260} value of the diffusible material formed per 1 hr., decreased steadily with increasing time of incubation. The ratio Pu:Py increased from 0.94 to 1.10, whereas the (A+U) : (G+C) ratio decreased from 1.50 during the first hours of hydrolysis to 0.88 after 52 hr. This indicates that in the initial phase of hydrolysis of RNA by RNase IIA were released the oligonucleotides which had the Pu : Py ratio close to unity and a predominance of A+U, whereas the oligonucleotides which had a higher Pu : Py ratio and a predominance of G+C, were released during the later phase of hydrolysis. The "core" remaining in the dialysis bag contained about 60% G, 20% A, and about 10% each of C and U.

Terminal nucleotides. Preliminary studies on the digestion products obtained from yeast RNA by the action of RNase IIA and treatment with prostate phosphomonoesterase and KOH, indicated that U and C were the bases occurring most

Table 1

Base composition of the diffusible products formed from yeast RNA during digestion by RNase IIA

The solution of 20 mg. RNA and 0.5 mg. of enzyme in 2 ml. of 0.1 M-ammonium carbonate buffer, pH 7.0, were dialysed against 10 ml. of the same buffer, and at the indicated time the composition of the outer fluid was determined. For details see text.

Hydrolysis time (hr.)	E_{260}^{1cm} (% of original RNA)	Rate of hydrolysis (E_{260}^{1cm}/hr.)	Base composition (mole %)				Pu:Py ratio	(A+U):(G+C) ratio
			G	A	C	U		
5	14.6	0.68	19.6	28.7	20.4	31.2	0.94	1.50
10	13.8	0.64	22.0	30.1	21.2	26.5	1.09	1.31
21	20.7	0.44	13.7	32.0	27.5	26.5	0.85	1.42
32	13.5	0.28	26.6	20.8	20.6	32.2	0.90	1.13
45	12.2	0.22	26.5	25.8	22.9	25.8	1.08	1.04
52	5.2	0.17	32.8	20.0	20.7	26.9	1.10	0.88
Core (nondiffusible)	20		60	20	10	10		
Original RNA	100		27.0	26.3	20.9	25.8	1.14	1.06

frequently at the 3′-terminus. For more detailed determinations, the following procedure was applied.

Each sample of hydrolysate II of RNA, obtained after 15, 60 and 180 min. of digestion by RNase, was incubated for 20 hr. with 3.5 units of snake venom exonuclease at pH 5.0. The mixture was separated into nucleosides, mononucleotides and nucleoside diphosphates by continuous-flow electrophoresis. The results of

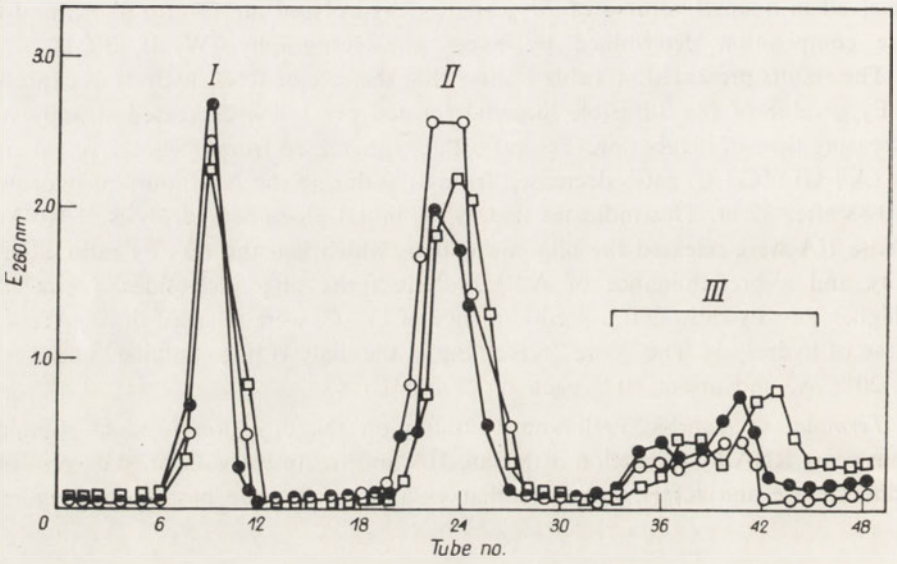

Fig. 10. Continuous-flow electrophoresis of hydrolysate II of yeast RNA, digested by RNase IIA for (○), 15 min.; (●), 60 min.; and (□), 180 min., and then treated with snake venom exonuclease. For details see Materials and Methods.

separation presented in Fig. 10 show that, regardless of the time of RNA digestion, three sharply separated fractions are obtained.

The amount of mononucleotides was slightly higher after 15 min. than after a longer time of digestion. This was to be expected because the average chain length of the initially formed fragments was greater than at later stages of hydrolysis. The results of quantitative analysis of the separated fractions are presented in Table 2. The fraction of nucleoside diphosphates contained exclusively C and U. Thus, only a pyrimidine base was present at the 3′-terminus. When the experiment involving 180 min. digestion was repeated, the same results were obtained, i.e. neither A nor G was found at the 3′-terminus.

An attempt was made to separate mononucleotides liberated by RNase IIA (3′-monophosphates) from those formed by the action of snake venom exonuclease (5′-monophosphates). A sample of the mononucleotide fraction (see Fig. 10) was freeze-dried, treated with purified snake venom 5′-nucleotidase (Sulkowski *et al.*, 1962), and then separated by electrophoresis under the same conditions as

Table 2

Composition of the double digest of RNA, first with RNase IIA (hydrolysate II) then with venom exonuclease

Fractions obtained in the experiment presented in Fig. 10 were pooled, freeze-dried, and then submitted to paper chromatography (Felix, Potter & Laskowski, 1960; Mukai, 1966) followed by paper electrophoresis (Markham & Smith, 1952). The nucleoside fraction represents the 5′-terminus, the diphosphonucleoside fraction, the 3′-terminus.

Time of digestion with RNase (min.)	I Nucleosides (μmoles)				II Mononucleotides (μmoles)				III Nucleoside diphosphates (μmoles)			
	A	C	G	U	pA	pC	pG	pU	pAp	pCp	pGp	pUp
15	0.79	0.15	0.73	0.25	1.08	0.91	1.60	1.27				
60	0.91	0.20	0.91	0.28	0.89	0.63	1.25	1.03				
180	0.90	0.20	1.00	0.23	0.94	0.52	1.21	1.14	0.0	0.67	0.0	1.35
180	0.81	0.21	1.03	0.28					0.0	0.70	0.0	1.90

for Fig. 10. Figure 11 (empty circles) shows a small peak of nucleosides which could have resulted only from 5′-mononucleotides. The latter, in turn, were derived from middle positions of the original fragments. In the nucleoside fraction, A and G, and a small amount of U, were identified by paper chromatography and spectral analysis. Another sample of the mononucleotide fraction was treated with crude *Crotalus adamanteus* venom containing 5′-nucleotidase and non-specific phosphatase, and fractionated by electrophoresis (Fig. 11, full circles). Large amounts of nucleosides and little of remaining mononucleotides were observed. In the nucleoside fraction, A, U, C, G, and I were found to be present, the last resulted from deamination of A.

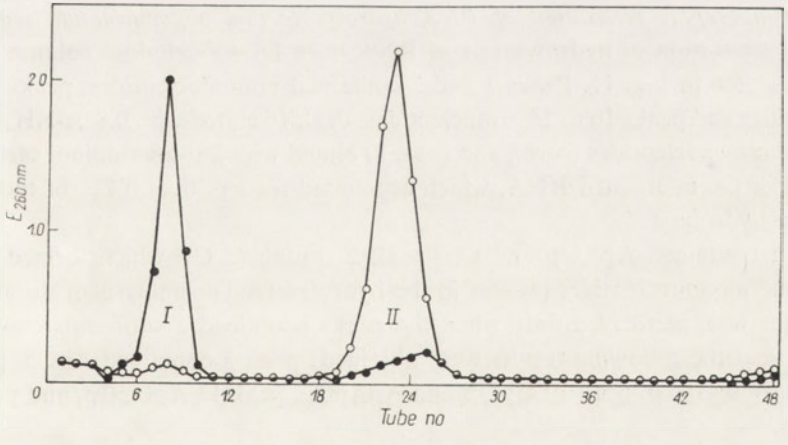

Fig. 11. Fractionation by continuous-flow electrophoresis of the peak of nucleotides from Fig. 10 treated with (○), purified snake venom 5′-nucleotidase, and (●), crude *Crotalus adamanteus* venom.

The presented experiments confirm the liberation of all four mononucleotides by RNase IIA. In oligonucleotides only pyrimidines are found in 3′-terminal position, whereas at the 5′-terminus, in addition to purine, a small amount of pyrimidine was also present. It follows that although RNase IIA has a specificity similar to that of pancreatic ribonuclease, the preference toward pyrimidines is less pronounced.

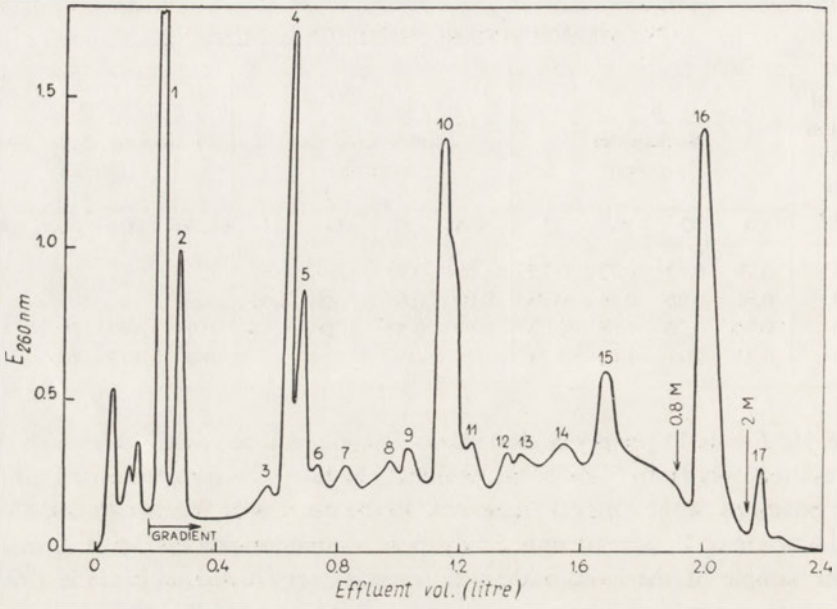

Fig. 12. Chromatographic separation of hydrolysate I of yeast RNA on DEAE-cellulose column in ammonium carbonate buffer, pH 8.6. A concentration gradient from 0.01 M to 0.04 M was applied, then the elution was carried out with 0.8 and 2.0 M-ammonium carbonate buffer solutions. For details see text.

Chromatographic separation of RNA hydrolysate and oligonucleotide sequence. A typical separation of hydrolysate I of RNA on a DEAE-cellulose column at pH 8.6 is presented in Fig. 12. Peaks 1 and 2 contained mononucleotides, peaks 3 to 9 dinucleotides and peaks 10 to 14, trinucleotides. Peak 16 eluted with 0.8 M-NH_4HCO_3 contained oligonucleotides (core), and peak 17 eluted with 2 M-ammonium carbonate consisted of the undigested RNA, which accounted for less than 0.7% of the RNA taken for hydrolysis.

Peak 1 contained Ap, Up and Cp; peak 2 contained Gp which formed about 30% of the mononucleotides present in the hydrolysate. The analysis of di- and trinucleotides was performed only when the peaks contained a sufficient amount of material, and the following results were obtained: peak 3 contained ApCp, peak 4 ApUp, peak 9 GpCp, peak 10 GpUp and ApApCp, peak 13 ApGpCp, and peak 14 GpApUp.

These sequences indicate that the oligonucleotides formed by the action of RNase IIA are of the type PupPyp, which confirms the results presented above.

DISCUSSION

Among autotrophic, chemosynthetic micro-organisms, of special interest are some sulphur bacteria which in order to assimilate CO_2 utilize the energy obtained from oxidation of elemental sulphur, hydrogen sulphite, or thiosulphate (Baalsrud, 1954; Vishniac & Trudinger, 1962). One of the strains of *Thiobacillus* was a subject of extensive studies (Peck & Fisher, 1962; Trudinger, 1964) aiming at elucidation of the mechanism of oxidation of thiosulphate to sulphate, as well as the mechanism of energy production in the course of this reaction.

In our previous studies on the metabolism of *Th. thioparus* (Szczepkowski & Skarżyński, 1952; Ostrowski, Skarżyński & Szczepkowski, 1954; Ostrowski & Krawczyk, 1957) it has been found that the cells of this microorganism possess a strong nucleolytic activity, resulting from the presence of three ribonucleases, a non-specific phosphodiesterase and phosphomonoesterase; some properties of these enzymes have been described (Ostrowski, 1961; Ostrowski & Walczak, 1961, 1963; Walczak & Ostrowski, 1964). The comparison of physico-chemical properties and the mechanism of action of the enzymes from autotrophic bacteria with those of analogous enzymes from other sources is of biological interest, therefore more detailed studies on the ribonucleases of *Th. thioparus* have been undertaken.

The results presented in this paper indicate that RNase IIA is an endonuclease highly specific with respect to the pentose and less specific with respect to the bases than pancreatic RNase. Yeast RNA is hydrolysed to 3′-oligonucleotides and to four 3′-mononucleotides, which form about 8% of the acid-soluble products. The "core" remaining after exhaustive digestion of yeast RNA, contains 60% guanine, 20% adenine and 10% each of cytosine and uracil. This composition of the limit polynucleotide suggests that RNase IIA does not digest longer fragments of RNA containing guanine as the predominating base in the sequence. RNase IIA is not a cyclizing nuclease since the cyclic nucleotides do not appear as intermediate products in the course of RNA digestion. This is also confirmed by the inability of RNase IIA to hydrolyse cyclic 2′,3′-CMP.

The presence of all four mononucleotides in the RNA hydrolysate may also indicate a lower specificity of RNase IIA in comparison with pancreatic RNase. However, it should be taken into account that in *Th. thioparus* cells two other ribonucleases, a non-specific phosphodiesterase and a phosphomonoesterase have been detected. They might have been present as contaminations in the RNase IIA preparation studied and, in that case, the detected mononucleotides could be due to the action of these enzymes, and their presence need not imply lower specificity of RNase IIA. This view is supported by the fact that the sequence of some di- and tri-nucleotides isolated from RNA hydrolysate is identical with that obtained by the action of pancreatic RNase. All the di- and tri-nucleotides studied had G or A at the 5′-end and C or U at the 3′-terminus. Thus it appears that, in the whole hydrolysate, the products obtained by RNase IIA digestion differ from those obtained with pancreatic ribonuclease, whereas the isolated individual oligonucleotides are in both cases the same.

Resistance to heating and low pH values, as well as ultracentrifugation data indicate that RNase IIA is a small protein, of molecular weight about 13 000, and also in this respect is similar to pancreatic RNase. However, the effect of ionic strength and of carboxymethylation on the activity of RNase IIA differ from those observed for pancreatic RNase. Therefore, on the basis of the so far obtained data on the specificity and physico-chemical properties of RNase IIA, it may be concluded that the enzyme from *Th. thioparus* is very similar to, but not identical with, pancreatic ribonuclease.

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WŁASNOŚCI I SWOISTOŚĆ RYBONUKLEAZY IIA Z *THIOBACILLUS THIOPARUS*

Streszczenie

1. Rybonukleaza IIA z *Thiobacillus thioparus* jest endonukleazą niecyklizującą o optimum działania przy pH 7.0, odporną na działanie wyższej temperatury i na działanie niskiego pH. Ciężar cząsteczkowy enzymu określony za pomocą wirowania w gradiencie sacharozy wynosi 13 500.

2. RNaza IIA uwalnia z RNA drożdżowego wszystkie cztery mononukleotydy (ok. 8% całości hydrolizatu), dwu- i trójnukleotydy z terminalnym fosforanem przy 3′-końcu, o sekwencji podobnej jak w przypadku RNazy trzuskowej, oraz pozostawia "core" zawierający ok. 60% guaniny.

3. Jako nukleotydy końcowe, od strony 5′-końca występują w znacznej przewadze zasady purynowe, a od 3′-końca — wyłącznie zasady pirymidynowe.

4. Porównanie własności fizykochemicznych i swoistości wykazuje, że RNaza IIA z *Th. thioparus* jest podobna do RNazy trzustkowej, ale nie jest z nią identyczna.

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ALICJA K. DRABIKOWSKA

ELECTRON TRANSPORT SYSTEM OF *SALMONELLA TYPHIMURIUM* CELLS

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, *Warszawa* 12, *Poland*

1. Intracellular distribution of the respiratory chain in *Salmonella typhimurium* has been investigated. The respiratory chain system was found mainly in the particulate fraction obtained from cells disrupted by ultrasonic treatment and sedimented at 105 000 *g*; it comprised succinate dehydrogenase, NADH dehydrogenase, ubiquinone, cytochromes a_1, a_2 and b_1, and probably cytochrome *c*. 2. Relative molar ratios of cytochrome a_2, cytochrome *b*, cytochrome *c*, flavoprotein and ubiquinone were 1:4:3:9:17. 3. The particulate fraction was able to reduce mammalian cytochrome *c* in the presence of succinate or NADH as substrates, whereas the 105 000 *g* supernatant fraction only in the presence of NADH. 4. The ubiquinone present in *Salmonella typhimurium* is of the same type as that of *Escherichia coli*, namely Q-8.

In the course of our investigations (Drabikowska, 1969) on the function of ubiquinone in *Salmonella typhimurium* cells the observation has been made that the particulate fraction, where almost total ubiquinone is localized, catalysed the oxidation of NADH and succinate with oxygen as the electron acceptor. Ubiquinone was found to undergo oxidoreductive changes during the oxidation of both these substrates.

Little is known about other electron transport components functioning within these organisms. Richmond & Maaløe (1962) showed that *Salmonella typhimurium* could exist either with or without cytochromes depending on the carbon source present in the medium. The bacteria grown on metabolites of the tricarboxylic acid cycle contained cytochromes but in those grown on glucose the concentration of cytochromes was very low or they were completely absent.

It was therefore supposed that an investigation of electron transport system in transformable *Salmonella* species may give a clearer understanding of the genetic control of the formation of the electron-transport system and its function.

The present investigation is an extension of the earlier studies and provides further data concerning the components of respiratory system of *Salmonella typhimurium*.

METHODS AND MATERIALS

Growth of bacteria. Salmonella typhimurium wild type strain LT-2 was grown in the medium *E* of Vogel & Bonner (1956) supplemented with trace elements as described previously (Drabikowska, 1969). The culture was aerated by 1 volume of air per 1 volume of the medium per minute. The air was heated to 37° before it passed through the medium. The cells were harvested by centrifugation at the beginning of the stationary phase of growth and washed once with 0.25 M-sucrose - 50 mM-tris solution, pH 7 (sucrose medium).

Preparation of subcellular fractions. The cells from 5 l. of the culture were suspended in about 50 ml. of sucrose medium, divided into two portions, placed in an ice bath and exposed to sonic oscillation for 10 min. in MSE-20 Kc sonicator at maximum output. The sonicated suspension was centrifuged at 3000 *g* for 15 min. to remove unbroken cells. In earlier work (Drabikowska, 1969) particles I and II were sedimented by centrifugation at, respectively, 18 000 and 105 000 *g*; the two kinds of particles had the same concentration of respiratory components as measured spectrophotometrically, and the same concentration of ubiquinone. Therefore in the present work the cell-free turbid supernatant was centrifuged directly at 105 000 *g* for 1 hr.

The sedimented particles were suspended in the sucrose medium, or in phosphate buffer if they were to be used for spectrophotometric determinations. The 105 000 *g* supernatant was used directly or after freezing and thawing.

Difference spectra. The difference spectra (reduced *minus* oxidized) of particulate and supernatant fractions were obtained in Unicam SP-800 spectrophotometer with the extension factor of 5 or 10, depending on protein concentration. The measurements were carried out at room temperature. Each cuvette contained 3 ml. of the preparation, which was vigorously aerated to exhaust endogenous substrates which might keep the respiratory components in the reduced state. Prior to registering the difference spectra, to one of the cuvettes a few crystals of natrium dithionite or substrate was added as reducing agent.

The concentration of individual components was calculated from their molar extinction coefficients. The following coefficients and wavelengths were used: cytochrome b_1, 17 500 at 562-575 nm (Deeb & Hager, 1964); cytochrome *c*, 17 300 at 554 - 540 nm (Tissières, 1956); cytochrome a_2, 8500 at 630 - 615 nm (White, 1965) and flavoprotein, 11 000 at 460 - 515 nm (Chance & Williams, 1955).

Measurements of oxidase activities. Oxygen uptake was measured polarographically with Clark electrode at room temperature in samples of 1.7 ml. The composition of media is described in legends to tables and figures. The initial concentration of dissolved oxygen in equilibrium with atmospheric oxygen was assumed to be 240 μM.

Cytochrome c *reduction.* The reduction of mammalian cytochrome *c* by subcellular fractions of *Salmonella typhimurium* in the presence of substrates was

measured in the recording Eppendorf photometer at 546 nm. The activity was calculated as described previously (Drabikowska & Szarkowska, 1965).

Identification of ubiquinone. The identification of ubiquinone was carried out by paper chromatography.

Determination of protein. Protein was estimated by the procedure described by Szarkowska & Klingenberg (1963).

Chemicals. NADH was obtained from Sigma Chem. Comp. (St. Louis, Mo., U.S.A.). Natrium dithionite ($Na_2S_2O_4$) was a product of Merck A. G. (Darmstadt, West Germany). Tris(hydroxymethyl)aminomethane was from Fluka A. G. (Buchs S. G., Switzerland). Other chemicals were of Polish origin.

RESULTS

NADH and succinate oxidases. The distribution of the two oxidases in subcellular fractions of *Salmonella typhimurium* is shown in Table 1. The particulate fraction oxidized both NADH and succinate. However, the activities of these oxidases differed from each other considerably. The rate of NADH oxidation was about ten times as high as that of succinate if measured in tris or phosphate medium (Table 2) and was practically independent of the medium used (sucrose-tris, tris-Cl, or phosphate) and pH value over the range 7 to 8.4.

The activity of succinate oxidase of the particulate fraction was dependent upon pH value (Fig. 1). A maximum in tris-Cl buffer was found at pH 8.4. The lower activity of succinate oxidation in the sucrose medium seems to be due to low pH rather than to the composition of the medium. The activity at pH 7.0 amounted to only about 30% of that at pH 8.4.

The supernatant fraction showed only some slight oxidation activity of NADH and succinate corresponding to about 3 - 8% of that observed in the particulate fraction (Table 1).

Table 1

Intracellular distribution in Salmonella typhimurium *of enzymes responsible for the oxidation of succinate and NADH*

The medium contained: 0.25 M-sucrose, 0.05 M-tris-Cl, pH 7, 0.2 - 0.4 mg. of protein of the particulate fraction or 10 - 20 mg. of protein of the supernatant fraction. Total volume was 1.7 ml. Substrates were added in the volume of 0.085 ml. to give the final concentration of 10 mM and 1 mM for succinate and NADH, respectively. Measurements were carried out at room temperature.

Fraction	Substrate	Oxygen uptake (μmoles/g. protein/min.)		Ratio of oxidation (NADH): (succinate)	
		Expt. A	Expt. B	Expt. A	Expt. B
Particulate 105 000 *g*	NADH	216	210	31	26
	succinate	7	8		
Supernatant	NADH	8	16	27	32
	succinate	0.3	0.5		

The rate of oxygen uptake by the particulate fraction with NADH and succinate was constant within the time of measurement (Fig. 2) which is typical of systems respiring *via* cytochrome oxidase (White, 1965). The same course of reaction with

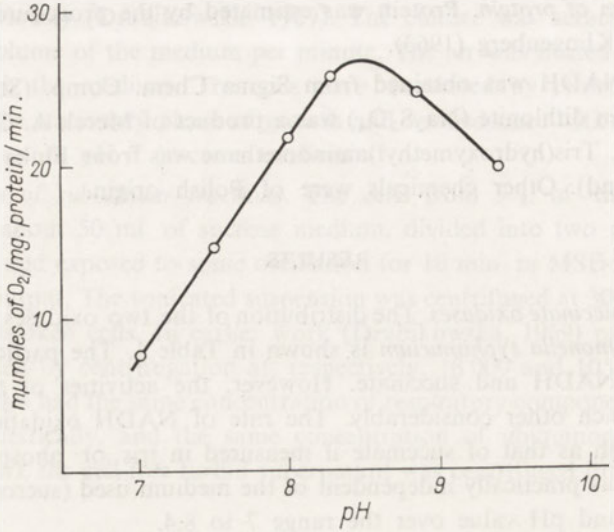

Fig. 1. Effect of pH on succinate oxidation by the particulate fraction of *Salmonella typhimurium*. The reaction medium contained in a volume of 1.7 ml.: 85 μmoles of tris-Cl buffer, 3.4 mg. of protein and 8.5 μmoles of succinate.

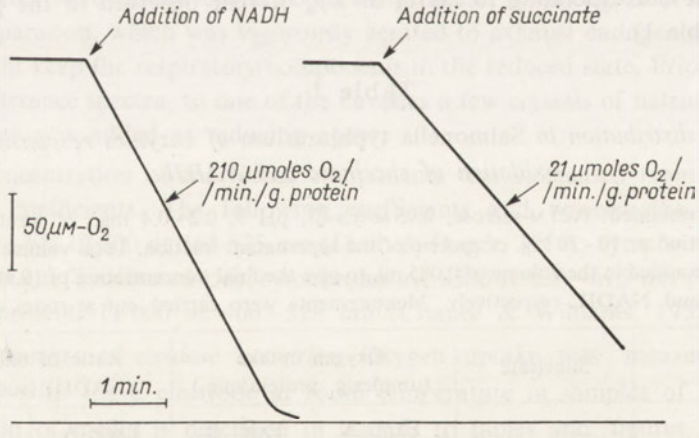

Fig. 2. Oxygen uptake by the particulate fraction of *Salmonella typhimurium*. Oxygen uptake was measured in 50 mM-tris-Cl medium of pH 8.4; 1.7 ml. of the medium contained 0.98 and 4.94 mg. of protein for NADH and succinate, respectively. The reaction was started by adding 1 mM-NADH or 10 mM-succinate and continued until all oxygen was exhausted.

Table 2

Oxidation rates of NADH and succinate by the particulate fraction of Salmonella typhimurium *under various experimental conditions*

Conditions employed (except the composition of medium) were as described in the legend to Table 1.

Reaction medium	Substrate	Oxygen uptake (μmoles/g. protein/min.)	Ratio of oxidation (NADH):(succinate)
0.25 M-sucrose - 0.05 M--tris-Cl, pH 7.0	NADH	216	31
	succinate	7	
0.05 M-tris-Cl, pH 8.4	NADH	210	10
	succinate	21	
0.05 M-phosphate buffer, pH 8	NADH	190	8
	succinate	24	

both these substrates was observed when the supernatant was used as the source of enzymes.

Spectrophotometric studies of subcellular fractions. Figure 3 demonstrates the difference spectra ($Na_2S_2O_4$ reduced *minus* oxidized), taken at room temperature, of the particulate and the supernatant fractions of *Salmonella typhimurium* cells grown aerobically.

The difference spectrum of the particulate fraction (Fig. 3A) taken at a high suspension density exhibited a strong absorption band at 430 nm and a shoulder at about 440 nm corresponding to γ band of cytochromes (Soret region). The minimum at 460 nm is characteristic of flavoproteins and the peak at 530 nm represents the β band of cytochromes *c* and *b*. In the region of α band a very characteristic peak of cytochrome b_1 at 562 nm can be seen. It is supposed that a small shoulder at 553-554 nm might be due to the absorption band of cytochrome *c*. A very small peak at 595 nm corresponds to a_1 type of cytochrome and that at 635 nm to cytochrome a_2.

The difference spectrum of the supernatant fraction (Fig. 3B) shows a very pronounced peak in the Soret region at about 420 nm and another one at 554 nm with a shoulder at 562 nm. This peak as well as that in the particulate fraction could not be resolved into two separate peaks, probably representing cytochromes *c* and *b*.

In both fractions, the same characteristic peaks of respiratory chain components appeared after the addition of NADH or succinate.

Molar concentrations of respiratory chain components, functioning in the *Salmonella typhimurium* particulate and supernatant fractions, are given in Table 3. It was assumed that the molar extinction coefficients of the respiratory carriers of *Salmonella typhimurium* are similar to those of carriers from other sources.

In the particulate fraction the concentration of ubiquinone was almost 17 times higher than that of cytochrome a_2. Cytochrome a_1 could not be quantitatively estimated because it appeared as only a very small peak.

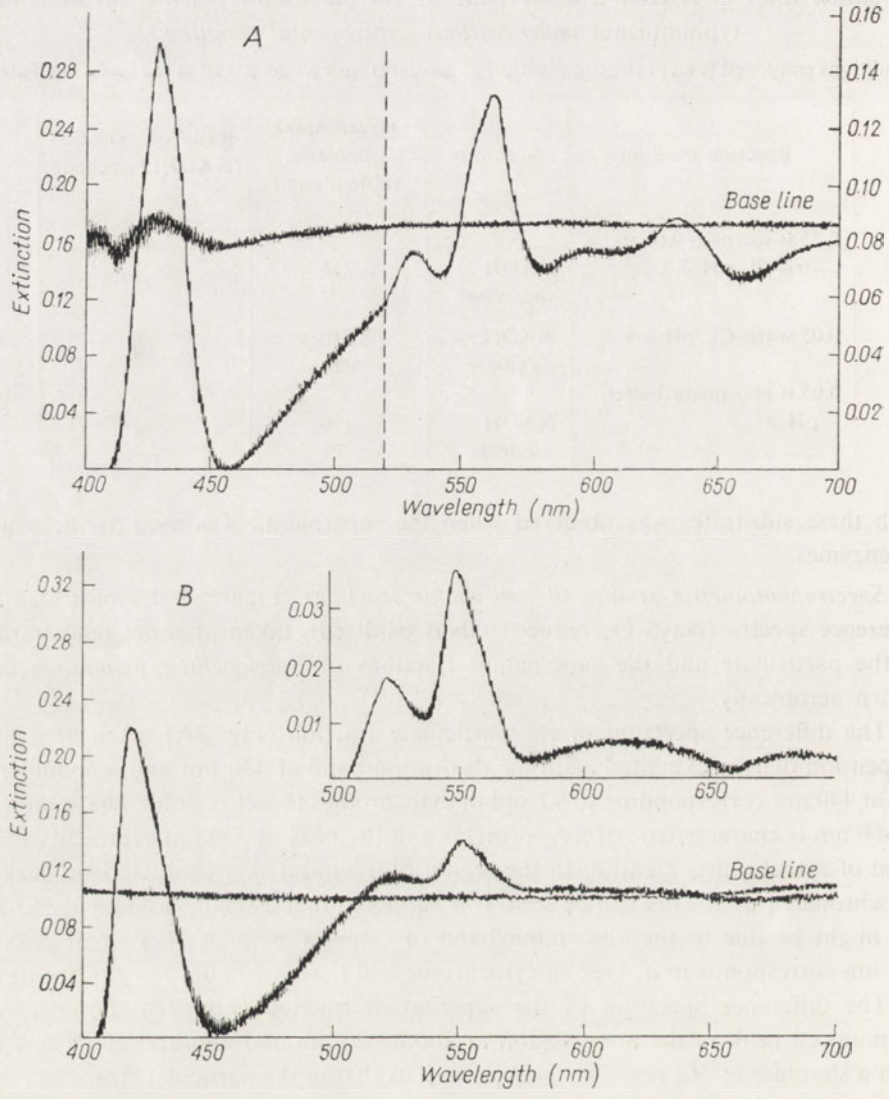

Fig. 3. Difference spectra (reduced *minus* oxidized) of *Salmonella typhimurium*: *A*, particulate fraction and *B*, supernatant. The reference system was aerated before measurement. The particulate fraction suspended in 0.1 M-phosphate buffer, pH 7.0, contained 8 mg. of protein/ml., whereas the supernatant contained 24.2 mg. of protein/ml. The measurements were carried out in 1-cm. cuvettes. The dashed perpendicular line in Fig. 3A indicates the change in extension factor from 5 (left-hand scale) to 10 (right-hand scale). The inset in Fig. 3B represents a part of the difference spectrum extended four times.

Table 3

Concentration of individual components of the respiratory system of Salmonella typhimurium

The concentrations of components were calculated on the basis of difference spectra using the molar extinction coefficients as described under Materials and Methods.

Component	Particulate fraction	Supernatant	Ratio of concentration (particulate): (supernatant)
	μmoles/g. protein		
Flavoprotein	1.19	0.31	3.9
Cytochrome *b*	0.47*	0.04*	11.8
Cytochrome *c*	0.33*	0.07*	4.7
Cytochrome a_2	0.13	not detectable	—
Ubiquinone	2.2	0.5	4.4

*Approximate values.

In the supernatant fraction the concentration of respiratory chain components was very low as compared with the particulate fraction. Some of the components could not be detected by spectrophotometric measurements, although from other experiments it follows that they must exist and function.

Reduction of mammalian cytochrome c. Particulate fractions obtained from *Salmonella typhimurium* cells were able to reduce mammalian cytochrome *c* with succinate and NADH as substrates (Table 4). This reduction, when measured in

Table 4

Reduction of exogenous cytochrome c *by particulate and supernatant fractions of* Salmonella typhimurium

The reaction medium contained: 50 mM-tris-Cl buffer (pH 8.0), 41 μM-mammalian cytochrome *c*, 2 mM-succinate or 0.6 mM-NADH and appropriate amount of the enzymic preparation in a total volume of 1 ml. Where indicated, 1 mM-KCN was added. The reaction was started by tipping in the substrate. The reduction of cytochrome *c* was measured as described in Materials and Methods.

Fraction	Additions	Cytochrome *c* reduction (μmoles/g. protein/min.)
Particulate	NADH	40
	NADH+KCN	44
	succinate	4
	succinate+KCN	3
Supernatant	NADH	160
	NADH+KCN	170
	succinate	0

tris medium of pH 8.0, was much lower than the uptake of oxygen. The reaction took place in the absence of KCN and its addition at that pH did not change the rate of reduction. After addition of NADH the reaction started immediately but the reduction with succinate was preceded by a lag phase of 3 to 5 min. After that time the reaction proceeded at a constant rate.

The supernatant fraction catalysed the reduction of cytochrome *c* only with NADH, but not with succinate, as substrate.

Considerable variations of reduction of cytochrome *c* were observed in both fractions, and were especially pronounced in the supernatant.

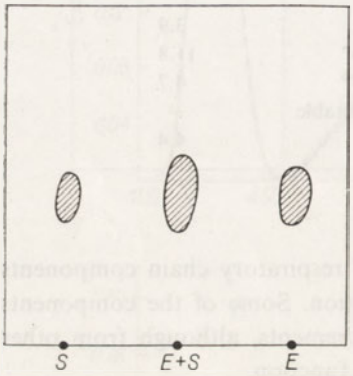

Fig. 4. Chromatogram of ubiquinones isolated from *Salmonella typhimurium* (*S*) and *Escherichia coli* (*E*). Whatman no. 1 paper was used. After development in 95% ethanol, the chromatogram was sprayed with ethanolic solution of reduced methylene blue.

Identification of ubiquinone. Chromatographic characteristics of the ubiquinone isolated from *Salmonella typhimurium* by the extraction method of Cox & Gibson (1966) were compared with those of ubiquinone from *Escherichia coli*. The R_F values of the two ubiquinones were found to be identical, and their mixture migrated as a single spot (Fig. 4).

DISCUSSION

Investigation of particulate and supernatant fractions of *Salmonella typhimurium* grown aerobically in a medium containing 0.2% citrate and 0.5% glucose as carbon sources showed that the oxidation processes occurred predominantly in the particulate fraction. Apart from succinate oxidase activity, it showed also a very high NADH oxidase activity which may serve as a system responsible for oxidation of NADH generated in processes occurring in cytoplasm.

On the basis of differential spectrum it was concluded that the oxidation system of *Salmonella typhimurium* consists of flavoproteins, cytochrome b_1, probably *c*-type cytochrome and cytochromes a_1 and a_2 which also occur in other bacteria and function as the terminal oxidase (Keilin, 1933; White, 1963, 1965).

It is supposed that the spectrum of cytochrome *c* which, at room temperature, does not show a separate peak, may contribute to a shoulder on the cytochrome b_1

peak. The observed asymmetry of the *a*-band of cytochrome b_1 at 562 nm might have been caused by the *a*-band of cytochrome *c* at 552-554 nm. According to Richmond & Kjeldgaard (1961) it may be assumed that this is cytochrome c_4 or c_5, or both, i.e. the same kind of cytochromes that occur in *Azotobacter vinelandii* (Jones & Redfearn, 1966).

Richmond & Maaløe (1962) showed that *Salmonella typhimurium* grown on components related to the Krebs cycle had a relatively high cytochrome content but the cells grown on components related to glucose contained no cytochromes at all or only very small amounts. Thus, it was expected that in Vogel's medium the cells would utilize only glucose. Citrate probably was not utilized, as the same strain cultivated on citrate under the same conditions exhibited but slight growth.

The presence of cytochrome *c* reductase in *S. typhimurium* (Table 4) may provide additional evidence for the occurrence of cytochrome *c* in these bacteria. Nevertheless, this problem could be solved only on the basis of low-temperature spectrum.

The characteristic changes of absorption spectra of cytochrome pigments which were observed also after addition of succinate or NADH lead to the recognition of the involvement of these cytochromes in the electron transport system of *Salmonella typhimurium*.

Although the cytochromes and flavoproteins of *S. typhimurium* are rather tightly bound to the membrane structures, it seems likely that they could be partly liberated into the supernatant by ultrasonic treatment. This would explain the oxidation activity found in the supernatant fraction. The amounts of some respiratory chain components might have been so small that it was impossible to detect them by spectrophotometric methods. These components may be the rate limiting step in the oxidation processes. The other components occurred in the supernatant in sufficient amounts to form well visible peaks in spectrophotometric measurements. A very marked peak occurred in the Soret region at 420 nm which reflects the *γ*-band of cytochrome *c*, and another peak at 554 nm, quite different in shape from that of the particulate fraction, could be observed. It may be supposed that the latter peak belongs to cytochrome *c* rather than to cytochrome *b*. Cytochrome *b*, unlike in the particulate fraction, may contribute at 562 nm to cytochrome *c* peak. The observed shift of the peak in the Soret region from 430 nm (particulate fraction) to 420 nm in the supernatant is probably due to the presence of cytochrome *c* as the dominant cytochrome component in this fraction.

Unfortunately, since cytochromes *b* and *c* did not form separate peaks, their molar concentrations could not be estimated precisely.

The fact that relative ratios of most of the respiratory chain components found in the particulate and the supernatant fractions were very similar may be an additional proof that the components found in the supernatant were derived from the membranes.

Preliminary investigations of the effect of KCN and 2-heptyl-4-hydroxyquinoline-*N*-oxide showed that the electron flow from substrates to oxygen may be inhibited

at the concentrations usually used. This effect is identical in respect to both fractions. These and other facts allow to conclude that the respiratory chain in *Salmonella typhimurium* is localized in the membrane fraction and involves the cytochrome system.

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UKŁAD PRZENOSZĄCY ELEKTRONY W KOMÓRKACH *SALMONELLA TYPHIMURIUM*

Streszczenie

1. Badano rozmieszczenie enzymów łańcucha oddechowego w komórkach *Salmonella typhimurium*. Okazało się, że we frakcji cząstek, otrzymanej po rozbiciu komórek ultradźwiękami i wirowaniu w 105 000 *g*, znajdują się: dehydrogenaza bursztynianowa, dehydrogenaza NADH, cytochromy a_1, a_2 i b_1, ubichinon i prawdopodobnie cytochrom *c*.

2. Względne stosunki molarne cytochromów a_2, b, c, flawoproteidów i ubichinonu wynoszą 1:4:3:9:17.

3. Frakcja cząstek katalizuje redukcję zwierzęcego cytochromu *c* w obecności bursztynianu i NADH jako substratów. Supernatant otrzymany po odwirowaniu cząstek katalizuje redukcję cytochromu *c* tylko w obecności NADH.

4. Ubichinon u *S. typhimurium* zawiera osiem jednostek prenylowych i jest identyczny z ubichinonem wyizolowanym z *Escherichia coli*.

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A. B. LEGOCKI, A. SZYMKOWIAK*, W. WIŚNIEWSKI** and J. PAWEŁKIEWICZ

PURIFICATION OF YELLOW LUPIN SEED tRNA's SPECIFIC FOR ISOLEUCINE AND SOME OTHER AMINO ACIDS

Department of Biochemistry, College of Agriculture, ul. Wołyńska 35, *Poznań, Poland*

1. tRNA's specific for isoleucine, leucine, valine, lysine and threonine were isolated from *Lupinus luteus* seeds by DEAE-Sephadex column chromatography, reversed-phase partition chromatography and Sephadex G-200 gel filtration. The presented method is simple, gives a high yield and should be useful for preparation of purified tRNA's from plant material. 2. Isoleucine- and leucine-specific tRNA's were separated into two components each. 3. The purity of the preparations obtained was as follows: isoleucine tRNA, about 82%; leucine and lysine tRNA's, 75%; valine tRNA, 52%, and threonine tRNA, 42%.

Our previous paper (Legocki, Szymkowiak & Pawełkiewicz, 1968) described the isolation of isoleucine-specific tRNA from yellow lupin seeds by consecutive DEAE-cellulose chromatography at elevated temperature, reversed-phase chromatography and countercurrent distribution, with a yield of about 5%. Now, we present a simpler and more efficient method which permits to prepare from the same material, in addition to isoleucine tRNA, also other tRNA species specific for leucine, valine, lysine and threonine. The procedure consists of DEAE-Sephadex chromatography, reversed-phase chromatography and finally gel filtration. Some properties of the preparations obtained are described.

MATERIALS AND METHODS

Reagents. ATP, GTP and GSH were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A). Chromosorb W, 60 - 80 mesh, acid washed, was a product of Matheson, Coleman & Bell (Norwood, Cinc., U.S.A.); dimethyldilaurylammonium chloride (Aliquat 204, lot no. 5, DW 348) was supplied by General Mills (Kankakee, III., U.S.A.); DEAE-Sephadex A-50 and Sephadex G-200 were purchased from Pharmacia (Uppsala, Sweden). Uniformly ^{14}C-labelled L-amino acids: lysine

* Present address: Department of General Chemistry, College of Agriculture, Poznań.
** Present address: Branch Department of General Chemistry, College of Agriculture, Bydgoszcz.

(88.7 mc/m-mole), threonine (61.4 mc/m-mole), leucine (79 mc/m-mole), isoleucine (79.3 mc/m-mole) and valine (96 mc/m-mole) were obtained from the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia). Other reagents were standard commercial products.

Crude tRNA from yellow lupin seeds (*Lupinus luteus*, var. Express) was isolated by the method of Ralph & Bellamy (1964) as described previously (Legocki, Szymkowiak, Pech & Pawełkiewicz, 1967) or with a modification consisting in application of NaCl extraction prior to phenol extraction. To 1 kg. of ground lupin seeds, 2 l. of 1 M-NaCl solution containing 0.5% sodium dodecyl sulphate was added at 0°. The suspension was mechanically shaken for 1 hr. at 0° and then centrifuged. To the supernatant one volume of liquefied phenol was added, shaken again for 15 min., centrifuged, and crude RNA was isolated as described previously (Legocki *et al.*, 1967). The use of NaCl extraction in the initial phase made the procedure considerably easier, particularly in large-scale tRNA preparation. However, isoleucine acceptor activity appeared to be somewhat lower in comparison to that obtained by direct phenol extraction.

The obtained crude tRNA was fractionated on DEAE-Sephadex column, and the isolated specific tRNA's were subjected to reversed-phase chromatography and gel filtration as described under *Chromatographic methods*.

Preparation of partially purified aminoacyl-tRNA synthetases. Lupin seed meal, 120 g., was extracted in a mortar with 360 ml. of 0.1 M-potassium phosphate buffer, pH 7.5, containing 0.4 M-sucrose, 0.01 M-magnesium chloride, 1 mM-mercaptoethanol and 10% of glycerol. The slurry was pressed through gauze, adjusted to pH 7.5 with 2 N-KOH and centrifuged at 65 000 *g* for 30 min. The supernatant was treated with ammonium sulphate, the fraction precipitated between 0.33 - 0.48 saturation was collected, dissolved in a small volume of 0.02 M-potassium phosphate buffer, pH 7.5, containing 1 mM-magnesium chloride, 1 mM-mercaptoethanol and 10% of glycerol, and dialysed overnight against 100 vol. of the same buffer. The dialysis residue was loaded into DEAE-cellulose column (2×16 cm.) equilibrated with the above buffer. The column was washed with 200 ml. of the same buffer and the fraction of aminoacyl-tRNA synthetases was eluted with the buffer containing additionally 0.25 M-NaCl. To the enzyme fraction, solid ammonium sulphate was added to 0.8 saturation and the mixture kept at -15° without loss of activity for several weeks. For assays, the protein precipitate was dissolved in a small volume of the above buffer and dialysed at 0° against the buffer overnight. The preparation clarified by centrifugation contained about 10 mg. of protein per 1 ml.

Assay for tRNA acceptor activity. The reaction mixture contained in a volume of 150 µl.: tris-HCl buffer (pH 7.3), 15 µmoles; KCl, 10 µmoles; ATP (disodium salt), 10 µmoles; GSH, 2 µmoles; ^{14}C-labelled amino acid (0.25 µc), 0.1 µmole; tRNA, 0.5 - 2.0 extinction units, and 0.2 mg. of synthetase preparation. After 20 min. at 35°, the tubes were transferred to the ice-bath, 100 µl. samples were withdrawn and applied to Whatman no. 3*MM* paper discs (2.4 cm. in diameter), which were then immersed in cold 5% trichloroacetic acid. After three changes of trichloroacetic acid at 15 min. intervals, the discs were washed in cold ethanol - ether mixture

(1:1,v/v) and finally in cold ether. The discs were air-dried and radioactivity was measured in 3 ml. of dioxane liquid scintillator according to Bray (1960) in the scintillation counter SE2 (BUTI, Warszawa, Poland). Control samples did not contain tRNA fraction.

Preparation of ^{14}C*-labelled aminoacyl-tRNA.* Aminoacylated tRNA's were prepared according to Moldave (1963) as described previously (Legocki *et al.*, 1967) using 80 extinction units of the purified tRNA preparations. The aminoacyl-tRNA's obtained had usually a specific activity of about 6000 counts/min./unit. They were fractionated by reversed-phase chromatography on Chromosorb W as described below.

Chromatographic methods. For preparation of DEAE-Sephadex column, to 600 g. of DEAE-Sephadex A-50 suspended in 25 l. of water, 500 g. of solid NaOH was gradually introduced under vigorous stirring. Sephadex was filtered on Buchner funnel and washed with water until the filtrate became neutral, then it was suspended in 22 l. of 1 M-NaCl solution and the pH was adjusted to 3.5 with 1 N-HCl. After filtration and exhaustive washing with water, the DEAE-Sephadex was suspended in 10 l. of 0.5 M-NaCl, and $MgCl_2$ and tris-HCl buffer of pH 7.4 were added to final concentrations of 0.01 and 0.02 M, respectively. The suspension was filtered and the Sephadex was resuspended in 10 l. of 0.5 M-NaCl - 0.01 M-$MgCl_2$ - 0.02 M-tris-HCl buffer, pH 7.4, and poured into a column (5×90 cm.). The crude tRNA preparation, 2 g., dissolved in 130 ml. of the above buffer solution was applied to the column. The elution was carried out with a linear NaCl concentration gradient using 5 l. of 0.02 M-tris-HCl buffer, pH 7.4, 0.02 M-$MgCl_2$ and 0.75 M-NaCl in the reservoir and 5 l. of 0.02 M-tris-HCl, pH 7.4, 0.01 M-$MgCl_2$ and 0.5 M-NaCl in the mixing chamber. The flow rate was about 100 ml./hr., 33.5 ml. fractions were collected, and the extinction measured at 260 mμ. The acceptor activity was determined in every other tube.

Reversed-phase chromatography was carried out as described by Kelmers, Novelli & Stulberg (1965) on dimethyldilaurylammonium chloride-coated Chromosorb W. The column (240×0.9 cm.) was equilibrated with 0.05 M-tris-HCl buffer, pH 7.3, saturated with isoamylacetate and containing 5 mM-$MgCl_2$ and 0.3 M-NaCl. The individual tRNA's obtained after DEAE-Sephadex chromatography were applied to the columns and eluted with 0.3 to 1.15 M-NaCl concentration gradient in 5 mM-$MgCl_2$ - 0.05 M-tris-HCl buffer, pH 7.3. Fractions of 7.5 ml. were collected, the flow rate being 0.8 ml./min. The recovery of u.v. absorbing materials was nearly 96%.

For analytical fractionation of ^{14}C-labelled aminoacyl-tRNA, 2.5 mg. of the preparation was applied to a Chromosorb W column prepared as above but of smaller diameter (0.6 cm.), eluted as described above and fractions of 3.5 ml. were collected. The aminoacyl-tRNA was precipitated with 10% trichloroacetic acid in the presence of 0.35 mg. of carrier yeast RNA, and radioactivity was measured as described previously (Legocki *et al.*, 1967).

Gel filtration on Sephadex G-200 was carried out on a column (100×1.8 cm.) equilibrated with 5 mM-$MgCl_2$ - 0.025 M-potassium acetate buffer, pH 7. The

individual tRNA fractions obtained after reversed-phase chromatography were precipitated with ethanol, 30 - 40 mg. was dissolved in 2 - 3 ml. of the equilibration buffer, dialysed, and applied to the column. Fractions of 2.5 ml. were collected and extinction measured at 260 mμ. The acceptor activity was determined in every other tube.

Analytical methods. The amount of RNA was determined taking $E^{1\%}_{260}=192$; the temperature profiles and nucleotide composition were examined as described previously (Legocki *et al.*, 1968).

RESULTS

Partial purification of specific tRNA's by DEAE-Sephadex column chromatography. For fractionation of lupin tRNA on DEAE-Sephadex, 8 g. of the crude tRNA preparation was used, four separate chromatographic runs being performed with 2 g. portions of the material. The recovery of ultraviolet-absorbing materials from the column amounted to about 90%.

The acceptor activity for isoleucine was eluted in three unequal peaks, two of which were incompletely resolved (Fig. 1a). This result is in agreement with the

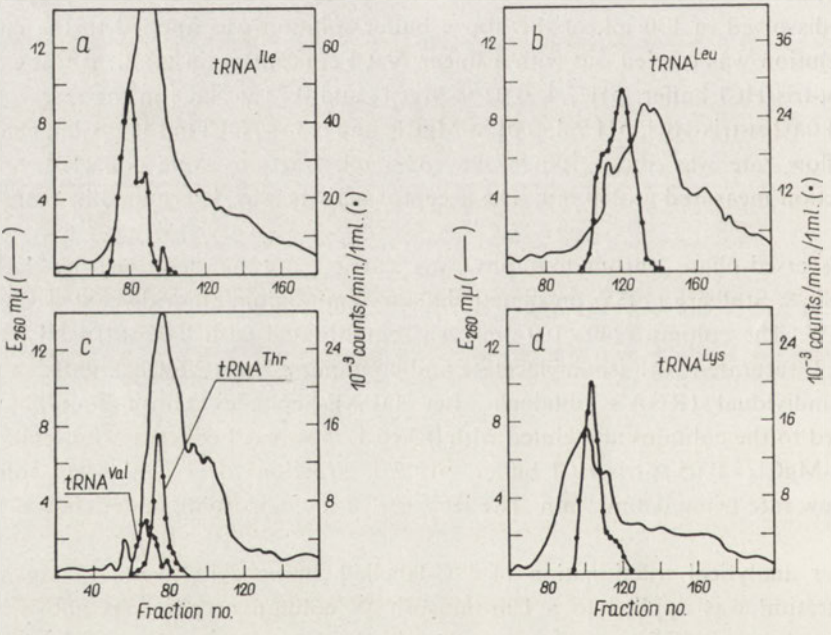

Fig. 1. DEAE-Sephadex A-50 column chromatography of crude tRNA from yellow lupin seeds. Samples containing each 2 g. of crude tRNA were applied to columns (90×5 cm.) and eluted with a linear NaCl concentration gradient from 0.5 to 0.75 M, in 0.01 M-$MgCl_2$ - 0.02 M-tris-HCl buffer, pH 7.4. Fractions of 33.5 ml. were collected, and absorption of each fraction was measured at 260 mμ. Acceptor activity for the [^{14}C]amino acids was determined as described in the text: (*a*), for isoleucine; (*b*), for leucine; (*c*), for valine and threonine; (*d*), for lysine.

previously obtained data on the fractionation of crude lupin tRNA on hydroxyapatite column (Legocki & Pawełkiewicz, 1967) and by reversed-phase chromatography (Legocki *et al.*, 1968). Fractions 71 - 91 containing the two major peaks of isoleucine-tRNA were pooled and used for further purification. The recovery of acceptor activity at that stage amounted to 52% of that of the initial crude preparation.

All the acceptor activity for leucine was eluted as a single but unsymmetrical peak (Fig. 1b), which is consistent with the heterogeneity of $tRNA^{Leu}$, previously observed on hydroxyapatite chromatography (Legocki & Pawełkiewicz, 1967). The $tRNA^{Leu}$ preparation was purified 4.6 times, the yield being 58%.

Valine acceptor activity was eluted, similarly as isoleucine- and leucine-specific tRNA's, at the front of the u.v. absorption peak. The acceptor activity curve showed the presence of two components (Fig. 1c). A similar elution pattern was obtained for [^{14}C]valyl-tRNA by chromatography on protamine-coated kieselguhr (Legocki *et al.*, 1967). As one of the components appeared in very small amounts, it was not used for further purification. The yield of valine tRNA was rather low and amounted to 27%.

Threonine and lysine acceptor activities (Figs. 1c and 1d) were eluted later in relation to the front of the u.v. absorption peak although they partially overlapped the elution volume of other tRNA's. Peaks of these two tRNA's were almost symmetrical, with only quite small shoulders. At this stage, the $tRNA^{Thr}$ and $tRNA^{Lys}$ preparations were purified 6.7 and 9.3 times, respectively.

Reversed-phase column chromatography. The tRNA fractions obtained by DEAE-Sephadex column chromatography, were subjected to reversed-phase column chromatography on Chromosorb W.

Isoleucine acceptor activity was found in two peaks (Fig. 2a), designated $tRNA_I^{Ile}$ and $tRNA_{II}^{Ile}$. The two tRNA's (fractions 50 - 58 and 63 - 71, respectively) contained together 47% of the total isoleucine tRNA initially present in the crude lupin tRNA preparation, and 90% of the $tRNA^{Ile}$ present in the preparation after DEAE-Sephadex chromatography. Their acceptor activities amounted to 22 and 21 mμmoles of isoleucine/mg. tRNA, respectively. Thus, at this stage the purity of both species increased 14 times, and in these fractions nearly 60% of RNA molecules could be charged with isoleucine. The charging of tRNA was calculated from the uptake of radioactivity in the process of aminoacylation; the molecular weight of tRNA was assumed to be 27 000, thus 1 mg. could accept 37 mμmoles of amino acid.

Leucine-specific tRNA was separated into two major peaks (Fig. 2b) which were designated $tRNA_I^{Leu}$ (fractions 60 - 76) and $tRNA_{II}^{Leu}$ (fractions 80 - 90). The third, very small peak of $tRNA_{III}^{Leu}$ was rejected. Acceptor activities of $tRNA_I^{Leu}$ and $tRNA_{II}^{Leu}$ preparations were 22.6 and 17 mμmoles of leucine per 1 mg. of RNA, respectively.

Valine, threonine and lysine acceptor activities emerged from the column as single, almost symmetrical peaks (Fig. 2c, d, e), indicating homogeneity of the preparations. The purity of valine- and threonine-specific tRNA's, however, was much lower than that of isoleucine or leucine tRNA's and the acceptor activity did

not exceed 12 mμmoles of the amino acid per 1 mg. of RNA. The purification of lysine tRNA was somewhat higher, nearly 18-fold.

Purification of specific tRNA's by Sephadex G-200 gel filtration. To remove high-molecular-weight nucleic acids as well as their low-molecular-weight degradation products, the fractions from reversed-phase chromatography were precipitated with ethanol, dialysed, and applied to a Sephadex G-200 column. The results presented in Fig. 3 indicate that all the tRNA preparations contained considerable amounts of u.v. absorbing impurities. After gel filtration the acceptor activity of $tRNA_{II}^{Ile}$ increased to 31 mμmoles of isoleucine per 1 mg. of RNA, that is more than 46% in relation to the unfiltered product. Likewise the purity of other preparations increased by 16% for $tRNA_{I}^{Leu}$ and even by 67% for $tRNA^{Val}$.

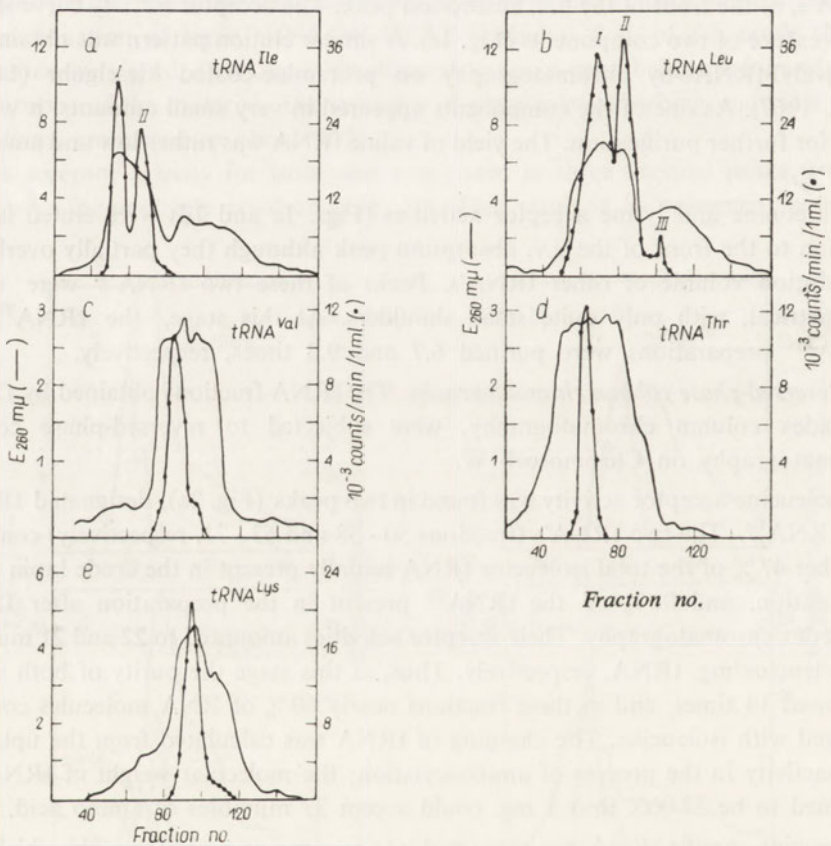

Fig. 2. Reversed-phase column chromatography on Chromosorb W coated with dimethyldilauryl-ammonium chloride, of tRNA fractions obtained after DEAE-Sephadex column chromatography (Fig. 1 *a-d*). Each sample was applied to a column (240×0.9 cm.) and eluted with a linear NaCl concentration gradient of 0.3 - 1.15 M in 5 mM-$MgCl_2$ - 0.05 M-tris-HCl buffer, pH 7.3, saturated with isoamyl acetate. Fractions of 7.5 ml. were collected. Absorption and acceptor activities for appropriate amino acids were determined as described in the text. (*a*), Isoleucine tRNA, fractions 71 - 91 (see Fig. 1); (*b*), leucine tRNA, fractions 105 - 130; (*c*), valine tRNA, fractions 60 - 73; (*d*), threonine tRNA, fractions 74 - 82; and (*e*), lysine tRNA, fractions 99 - 110.

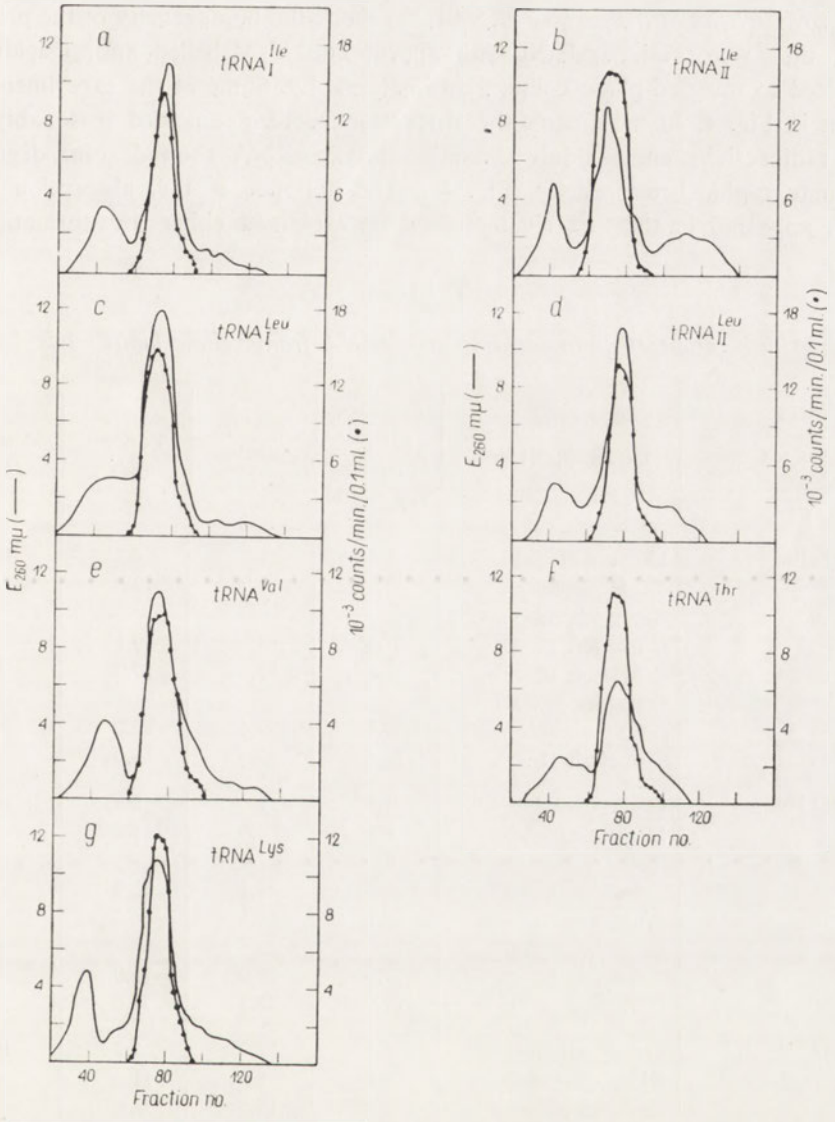

Fig. 3. Sephadex G-200 gel filtration of purified tRNA preparations. The tRNA's (30 - 40 mg. of RNA) obtained by reversed-phase chromatography (see Fig. 2, *a-e*) and dissolved in 5 mM-$MgCl_2$ - 0.025 M-potassium acetate buffer were filtered each through a Sephadex G-200 column (100 × 18 cm.). Fractions of 2.5 ml. were collected, and absorption and acceptor activity were determined as described in the text. (*a*), $tRNA_I^{Ile}$, fractions 50 - 58 (see Fig. 2); (*b*), $tRNA_{II}^{Ile}$, fractions 63 - 71; (*c*), $tRNA_I^{Leu}$, fractions 60 - 76; (*d*), $tRNA_{II}^{Leu}$, fractions 80 - 90; (*e*), $tRNA^{Val}$, fractions 80 - 90; (*f*), $tRNA^{Thr}$, fractions 60 - 68, and (*g*), $tRNA^{Lys}$, fractions 89 - 100.

Recovery of acceptor activities and degree of purification attained at the successive steps of the procedure, are summarized in Table 1.

Characteristics of the purified tRNA's. To check the homogeneity of the preparations, they were aminoacylated with appropriate ^{14}C-labelled amino acids and subjected to reversed-phase column chromatography. Some of the experiments are shown in Fig. 4. In most cases the absorption profiles coincided reasonably well with radioactivity curves; only threonine-specific tRNA showed some degree of chromatographic heterogeneity (Fig. 4f). The presence of u.v. absorption peaks which appeared in the first eluates could be explained either by formation of

Table 1

Purification of amino-acid specific tRNA from yellow lupin seeds

tRNA	Purification stage	Acceptor activity (mμmoles/ /mg. RNA)	Purification factor	Purity (%)	Yield (%)
$tRNA^{Ile}$	Crude material	1.5	1	4.2	100
	DEAE-Sephadex	7.3	4.7	19.8	52
	Reserved-phase				
	fraction I	21.9	14	59.1	47
	fraction II	21.1	13.5	57.0	
	Sephadex G-200				
	fraction I	30.1	19.4	82.0	32
	fraction II	30.8	20.0	83.0	
$tRNA^{Leu}$	Crude material	1.1	1	2.8	100
	DEAE-Sephadex	4.8	4.6	13.0	58
	Reversed-phase				
	fraction I	22.6	19.1	53.9	50
	fraction II	17.1	16.5	46.2	
	Sephadex G-200				
	fraction I	27.0	26.0	73.0	44
	fraction II	28.5	27.5	77.0	
$tRNA^{Val}$	Crude material	0.9	1	2.2	100
	DEAE-Sephadex	4.1	5.0	11.1	27
	Reversed-phase	12.2	14.5	32.9	18
	Sephadex G-200	19.5	24.0	52.7	16
$tRNA^{Lys}$	Crude material	1.0	1	2.7	100
	DEAE-Sephadex	9.0	9.3	25.2	67
	Reversed-phase	17.8	17.8	48.1	43
	Sephadex G-200	27.9	27.9	75.3	41
$tRNA^{Thr}$	Crude material	0.4	1	1.1	100
	DEAE-Sephadex	3.5	6.7	7.4	87
	Reversed-phase	11.1	27.2	30.0	57
	Sephadex G-200	15.7	38.5	42.4	34

unreactive aggregates during the chromatography, or by incomplete charging of tRNA's with amino acids, with subsequent resolution of the charged and uncharged components.

Chromatographic homogeneity is not a direct proof of purity of tRNA. By direct aminoacylation it was found that the lysine tRNA preparation contained at least 2% of isoleucine tRNA. Similarly other preparations contained 5 - 10% of foreign tRNA's.

The nucleotide composition of the isolated tRNA preparations is presented in Table 2. The sum of guanine and cytidine nucleotides was very similar in all

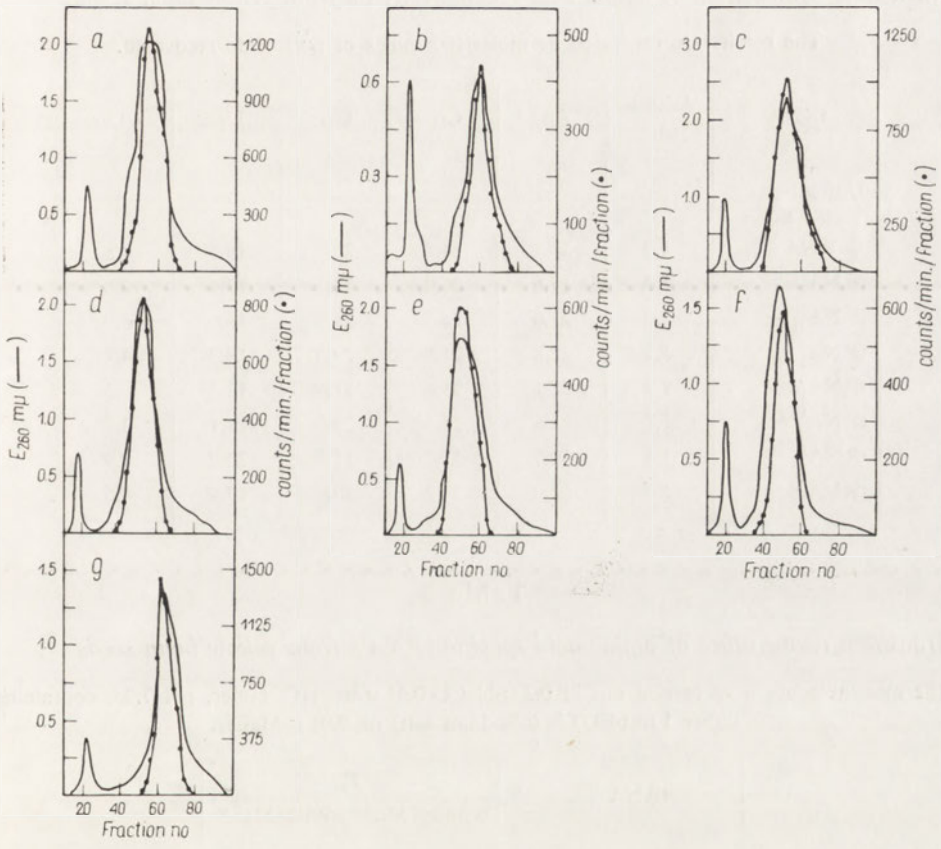

Fig. 4. Reversed-phase column chromatography of [^{14}C]aminoacyl-tRNA. The tRNA preparations after Sephadex G-200 gel filtration (fractions 64 - 82, see Fig. 3, *a-g*) were aminoacylated with appropriate amino acids as described under Methods. Samples of 2.5 mg. of the individual aminoacyl-tRNA's were applied to the column (240×0.6 cm.) of Chromosorb W coated with dimethyldilaurylammonium chloride, and eluted with a 0.3 - 2.7 M-NaCl concentration gradient in 5 mM-$MgCl_2$ - 0.05 M-tris-HCl buffer, pH 7.25; 3.5 ml. fractions were collected and radioactivity measurements were carried out as described under Methods. (*a*), Isoleucyl-tRNA$_I^{Ile}$; (*b*), isoleucyl-tRNA$_{II}^{Ile}$; (*c*), leucyl-tRNA$_I^{Leu}$; (*d*), leucyl-tRNA$_{II}^{Leu}$; (*e*), valyl-tRNA^{Val}; (*f*), threonyl-tRNA^{Thr}; (*g*), lysyl-tRNA^{Lys}.

preparations with the exception of $tRNA_I^{Ile}$. Both the two isoleucine tRNA species and the two leucine tRNA species differed also in the content of pseudouridylate; $tRNA_I^{Ile}$ had the lowest content (2.5%) and $tRNA_{II}^{Leu}$ the highest (6.3%), whereas intermediate values were obtained for tRNA's specific for threonine, valine and lysine.

The melting temperature (Table 3) showed the known shift depending on the absence or presence of magnesium ions which stabilize the rigid structure of RNA.

Table 2

Nucleotide composition of amino acid-specific tRNA's from yellow lupin seeds

The results are expressed as moles/100 moles of nucleotide recovered.

tRNA	Adenosine	Ap	Cp	Gp	Up	ψUp
Unfractionated lupin tRNA	1.0	22.1	24.5	28.8	18.5	4.8
$tRNA_I^{Ile}$	1.2	17.6	28.0	33.2	17.4	2.5
$tRNA_{II}^{Ile}$	1.0	21.0	24.5	29.9	18.6	4.9
$tRNA_I^{Leu}$	1.2	22.8	24.0	29.3	18.3	4.4
$tRNA_{II}^{Leu}$	1.0	22.1	25.8	31.6	13.2	6.3
$tRNA^{Val}$	1.2	23.6	25.3	29.0	17.1	3.7
$tRNA^{Lys}$	1.3	20.7	22.2	31.7	20.2	3.9
$tRNA^{Thr}$	1.0	22.0	24.2	30.9	17.4	4.5

Table 3

Transition temperature of amino-acid specific tRNA's from yellow lupin seeds

The measurements were carried out in 0.2 M-NaCl - 0.01 M-tris-HCl buffer, pH 7.25, containing either 1 mM-EDTA (disodium salt) or 0.01 M-$MgCl_2$.

tRNA	T_m	
	Without Mg^{2+}	With Mg^{2+}
$tRNA_I^{Ile}$	47	71
$tRNA_{II}^{Ile}$	50	58
$tRNA_I^{Leu}$	51	73
$tRNA_{II}^{Leu}$	54	68
$tRNA^{Val}$	50	64
$tRNA^{Lys}$	44	65
$tRNA^{Thr}$	40	58
Unfractionated tRNA	46	59

Considerable differences in melting temperatures between individual tRNA's were observed also in the presence of magnesium, the difference between $tRNA_I^{Ile}$ and $tRNA_{II}^{Ile}$ being as high as 13°.

DISCUSSION

As reported in this paper, isoleucine-, leucine- and lysine-specific tRNA's can be substantially purified by the use of three relatively simple procedures. The use of DEAE-Sephadex column chromatography, reversed-phase partition chromatography, and Sephadex G-200 gel filtration gave preparations of these specific tRNA's of about 75 - 80% purity and in a yield exceeding 32%. Although the first two methods were successfully used for purification of several specific tRNA's from *Escherichia coli* (Nishimura, Harada, Narushima & Seno, 1967; Ishikura & Nishimura, 1968), from yeast (Takeishi, Nishimura & Ukita, 1967) and from rat liver (Nishimura & Weinstein, 1969), with lupin tRNA they did not give satisfactory results. This was possibly due to lower purity of our crude preparations. However, the subsequent application of gel filtration permitted to obtain a high degree of purification of lupin seed tRNA's. Therefore it seems reasonable to expect that the method presented in this paper may be useful in work with plant material. It should be noted that the general procedure for isolation of specific amino acid tRNA published recently by Gillam, Blew, Warrington, Tigerstrom & Tener (1968) was found in this laboratory to be unsuitable for lupin tRNA's.

The analysis of nucleotide composition of isolated tRNA's showed that $tRNA_{II}^{Ile}$ had practically identical composition with the isoleucine tRNA preparation obtained previously and designated $tRNA_{2,3}^{Ile}$ (Legocki *et al.*, 1968). As reported in that paper, direct countercurrent distribution of crude tRNA gave three fractions, $tRNA_1^{Ile}$, $tRNA_{2,3}^{Ile}$ and $tRNA_4^{Ile}$. When DEAE-cellulose chromatography at 72° was applied as the first step of the purification procedure, on subsequent reversed-phase chromatography only $tRNA_{2,3}^{Ile}$ was obtained. The disappearance of fraction $tRNA_1^{Ile}$ was by us interpreted at that time as indicating that chromatography at elevated temperature removed or altered some isoleucine-specific tRNA species. By the presented method, $tRNA_I^{Ile}$ corresponding to the previously missing fraction $tRNA_1^{Ile}$ was obtained, thus confirming our previous supposition.

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OCZYSZCZANIE tRNA Z NASION ŁUBINU ŻÓŁTEGO, SPECYFICZNYCH DLA IZOLEUCYNY I INNYCH AMINOKWASÓW

Streszczenie

1. Stosując kolejno chromatografię na DEAE-Sephadex, rozdział w odwróconej fazie chromatograficznej oraz sączenie żelowe na kolumnie Sephadex G-200, izolowano z nasion *Lupinus luteus* tRNA specyficzne dla izoleucyny, leucyny, waliny, lizyny i treoniny. Opisana metoda jest prosta, pozwala uzyskać wysoką wydajność i powinna okazać się użyteczna dla otrzymywania specyficznych tRNA z materiału roślinnego.

2. tRNA specyficzne dla izoleucyny i leucyny rozdzielały się każdy na dwie frakcje.

3. Czystość preparatów tRNA specyficznych dla izoleucyny wynosiła ok. 82%, dla leucyny i lizyny ok. 75%, dla waliny 52%, a dla treoniny 42%.

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MARTA RYTEL, T. GOŁASZEWSKI and J. W. SZARKOWSKI

BIOSYNTHESIS OF RIBONUCLEIC ACID IN MITOCHONDRIA OF RYE (*SECALE CEREALE*) LEAVES *IN VITRO*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, *Warszawa* 12, *Poland*

1. Incorporation *in vitro* of [2-^{14}C]uracil into RNA of intact or disintegrated mitochondria of rye leaves, and into RNA of mitochondrial subfractions obtained by differential centrifugation, was studied. 2. When intact or disintegrated mitochondria were incubated with [2-^{14}C]uracil, the highest RNA activity was found in the 100 000 *g* pellet, whereas when the isolated subfractions were incubated, only RNA from the 100 000 *g* supernatant was highly labelled. 3. The results obtained indicate that the structural integrity of mitochondria is not an indispensable factor for incorporation of precursors into mitochondrial RNA. 4. The incorporation of L-[U-^{14}C]leucine into mitochondrial proteins was negligible.

In the last few years, the intensive studies carried out on mitochondrial nucleic acids were focussed on two main problems: involvement of nucleic acids in protein biosynthesis within the mitochondrion and participation of mitochondria in cell RNA and DNA anabolism. The latter problem is of importance in view of the fact that mitochondria constitute a semiautonomous system within the cell (Gibor & Granick, 1964; Rabinowitz, 1968).

It has been established (Luck & Reich, 1964; Wintersberger & Tuppy, 1965; Schweiger, Dillard, Gibor & Berger, 1967) that mitochondria are able to incorporate nucleotide precursors into their RNA. Intramitochondrial sites of RNA synthesis are, however, not known. The recent attempts at separation of defined morphological elements of mitochondria (Sottocasa, Kuylenstierna, Ernster & Bergstand, 1967; Schnaitman & Greenawalt, 1968) point to compartmentation of various enzymic activities within the organella. It seemed therefore interesting to check RNA metabolism at submitochondrial level.

Previously we have found that RNA is unevenly distributed within mitochondria isolated from primary rye leaves (Rytel, Szarkowski & Gołaszewski, 1969 b). To establish the location of RNA synthesis within the mitochondrion we have undertaken the studies on RNA turnover in mitochondrial subfractions. In this paper we present some results on the subject and we compare the RNA turnover of mitochondria with that of other cell organellae of rye leaves. Preliminary data have

been presented at the VIth FEBS Meeting (Szarkowski, Gołaszewski & Rytel, 1969). Some data on the incorporation of amino acids into mitochondrial proteins *in vitro* are also presented.

MATERIALS AND METHODS

Special reagents. [2-^{14}C]Uracil, 6.5 mc/m-mole (Division of Isotope Distribution, Institute for Nuclear Research, Warszawa, Poland); [6-^{14}C]orotic acid, 2.3 mc/m-mole (Hungarian Institute of Isotopes, National Atomic Energy Commission, Budapest, Hungary); L-[U-^{14}C]leucine, 89 mc/m-mole (Institute for Research, Production and Utilization of Radioisotopes, Prague, Czechoslovakia). The labelled leucine was used after tenfold dilution with D,L-[^{12}C]leucine (Fluka A. G., Buchs SG, Switzerland). Bovine serum albumin, Fraction V (Armour Laboratories, Kankakee, Ill., U.S.A.). Kieselguhr (Hyflo-Super-Cel, Vulcascot, West Germany). Trypsin (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.) was purified by 2-day dialysis against 0.001 N-HCl at 4°, the insoluble material was discarded by centrifugation, the supernatant was freeze-dried and stored at -20°; non-crystalline bovine pancreas deoxyribonuclease I, ADP and GTP (Sigma Chemical Company, St. Louis, Mo., U.S.A.); [^{12}C]amino acids (Calbiochem, Los Angeles, Calif., U.S.A.); 2,5-diphenyloxazole (PPO), 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) (Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.); other chemicals used were analytical grade of Polish origin.

Plants. Rye (*Secale cereale* var. "Włoszanowskie") was cultivated as described previously (Ombach, Gołaszewski & Szarkowski, 1966) in a day-night cycle (green plants) and in darkness (etiolated plants). After 14 days of growth, the primary leaves, about 12 cm. long, were taken for experiments.

Isolation of cell organellae. Primary leaves, 400 g. wet wt., were cut into 1 cm. fragments and homogenized with about 500 ml. of ice-cold medium *A* (0.25 M-sucrose - 10 mM-magnesium acetate - 10 mM-tris-HCl buffer, pH 7.3) for three periods of 30 sec. interspaced by 30 sec. cooling periods in a "Turmix" blender operating at maximum speed. The homogenate was filtered through four layers of cheese-cloth and subjected to differential centrifugation.

Nuclei and cell debris were removed by centrifugation at 600 *g* for 3 min. From the supernatant, plastids (chloroplasts from green leaves and etioplasts from etiolated leaves) were sedimented at 1000 *g* for 14 min. Crude plastids were resuspended in medium *A* and after preliminary centrifugation at 600 *g* for 3 min. the purified plastids were collected at 1000 *g* for 14 min.

Mitochondria were sedimented at 12 000 *g* for 15 min., resuspended in 40 ml. of medium *A* using a Teflon pestle and, after preliminary centrifuging at 1000 *g* for 14 min. to remove the plastidial impurities, the suspension was again centrifuged at 12 000 *g* for 15 min. The washing procedure was repeated twice with fresh portions of medium *A*.

Microsomes were collected by centrifugation at 100 000 *g* for 2 hr. (Spinco model L-2 ultracentrifuge no. 30 rotor).

Subfractionation of mitochondria. The washed mitochondrial pellet was suspended in 5 ml. of medium *A* (about 7 mg. of protein/ml.) and subjected to sonic oscillation (MSE, 100 W, ultrasonic disintegrator, 20 kcyc./sec.) for three periods of 1 min. interspaced by 30 sec. intervals at 2°. The preparation was made up to 20 ml. with medium *A* and subjected to differential centrifugation; three mitochondrial subfractions were isolated: 20 000 *g* pellet (for 20 min.); 100 000 *g* pellet (for 2 hr.); and 100 000 *g* supernatant.

To the supernatant (except for some experiments, as specified below) solid sodium chloride up to 0.1 M final concentration and 3 vol. of cold 96% (v/v) ethanol were immediately added and after 24 hr. the nucleoprotein precipitate was collected by centrifugation.

Ribonucleic acid labelling. Freshly obtained subcellular fractions were suspended in medium *A* (about 5 mg. of protein/ml.) and incubated for 1 hr. at 37° with 1.53 μmoles of [2-^{14}C]uracil (10 μc) or 4.35 μmoles of [6-^{14}C]orotate (10 μc) in a final volume of 2 ml. containing about 5 mg. of protein. The incorporation was stopped by rapid cooling to 2° and adding 2 vol. of 0.25 M-sucrose - 2 M-NaCl - 3.2 mM-uracil (non-radioactive) and 10 vol. of cold 96% (v/v) ethanol. The mixture was allowed to stand overnight at 2° and then centrifuged. The sediment was washed several times by suspending in a mixture containing 1 vol. of 3.2 mM-uracil (unlabelled) solution and 2 vol. of 96% ethanol. RNA from the final sediment was extracted by the phenol method according to Click & Hackett (1966) using 0.1 M-tris-HCl buffer, pH 7.3. Samples incubated in the presence of 0.3 M-perchloric acid were taken as controls.

In one set of experiments, the radioactivity of RNA isolated from individual mitochondrial subfractions was estimated. The following types of experiments were performed: (*a*), Intact mitochondria were incubated with [2-^{14}C]uracil, then subjected to sonic treatment, and subfractions were isolated. (*b*), Mitochondria were ultrasonically disrupted, then incubated with labelled uracil, and subfractions were isolated. (*c*), Isolated mitochondrial subfractions were incubated under the same conditions (prior to incubation, the 100 000 *g* supernatant was evaporated to a small volume in the Pleybold freeze-drying plant, GT 1.5).

The radioactivity of RNA was measured with an automatic liquid-scintillation spectrometer (Packard Tri-Carb model 3003, efficiency for ^{14}C, 80%). To 11 ml. of the scintillation liquid (3 g. PPO and 0.3 g. POPOP per 1000 ml. of toluene) 3.5 ml. of absolute ethanol and 0.35 ml. of RNA solution in water (about 200 μg. of RNA or its hydrolysis products) were added.

Protein labelling. Isolated mitochondria were suspended in the medium of Kroon (1963) consisting of 0.25 M-sucrose - 50 mM-KCl - 5 mM-$MgCl_2$ - 1 mM-EDTA - 30 mM-KH_2PO_4 - 50 mM-tris-HCl buffer, pH 7.4. One milliliter of the suspension (3 - 4 mg. of protein) was incubated for 2 hr. at 37° under shaking with 0.04 μmole of L-[U-^{14}C]leucine (0.36 μc) in the presence of 0.3 m-mole of GTP, 2 m-moles of ADP and 50 μl. of the mixture of eighteen [^{12}C]amino acids (Webster, 1955). The mixture contained 100 μg. of each amino acid per 1 ml., except tyrosine,

1 μg./ml. The final pH of the incubation mixture was adjusted to 7.4 with NaOH, and the final volume was about 1.1 ml. The reaction was stopped by adding 5 ml. of 5% (v/v) trichloroacetic acid, the mixture was heated at 90° for 15 min., the precipitate washed with ethanol-ether (3:1, v/v) and once with ether (Kroon, 1963). The washed final precipitate was plated on aluminium planchettes with concentrated formic acid (Ehrenstein & Lipmann, 1961), dried under an infrared lamp (250 W), and the radioactivity determined in a Nuclear Chicago model 1152 gas-flow counter.

In experiments in which the distribution of radioactivity in mitochondrial subfractions was studied, the mitochondria were incubated with L-[U-^{14}C]leucine as above, then subjected to sonic oscillations, 10 ml. of 0.04 mM-[^{12}C]leucine were added and the mixture left overnight at 2°. Then the mitochondrial subfractions were isolated and the radioactivity determined in the precipitated protein.

Separation of RNA on columns of methylated albumin adsorbed on kieselguhr. The MAK columns were prepared according to Mandell & Hershey (1960) with some modifications described previously (Rytel, Szarkowski & Gołaszewski, 1969a). To the column (2×6 cm.) up to 2 mg. of nucleic acid was applied in 10 ml. of equilibration buffer (50 mM-sodium phosphate buffer, pH 6.7). The separation was performed at 20°. The column was washed with the same buffer until the absorption of effluent at 260 mμ became constant. Low-molecular-weight RNA was eluted with 0.7 M-NaCl, and high-molecular-weight RNA with 1.3 M-NaCl in the starting buffer. The samples were evaporated to a small volume under vacuum at room temperature and radioactivity was measured.

Isolation and estimation of RNA. RNA was isolated by two methods. For MAK column chromatography, the isolation was carried out by the phenol method (Click & Hackett, 1966) with the use of 0.1 M-tris-HCl buffer, pH 7.3. The amount of RNA was estimated at 260 mμ using the coefficient 24.0/mg./ml./cm. For other experiments, the material was purified according to Nieman & Poulsen (1963). From the ether powders obtained, RNA was extracted with 1 N-perchloric acid for 6 hr. at 4° (Ogur & Rosen, 1950). The extract was neutralized with 1 N-KOH, the potassium perchlorate removed by centrifugation, the supernatant evaporated to a small volume and used for radioactivity determination and RNA estimation by the orcinol method according to Mejbaum (1939).

Determination of DNA in mitochondria. DNA was extracted with 0.5 M-perchloric acid at 70° for 15 min. and estimated spectrophotometrically according to Nieman & Poulsen (1963, 1967) at 265 mμ and 310 mμ.

Deoxyribonuclease treatment of mitochondria. The isolated mitochondria were suspended in 0.15 M-sucrose - 5 mM-magnesium acetate - 0.1 M-phosphate buffer, pH 6.5, and 0.9% (w/v) NaCl, and incubated for 30 min. at 37° with deoxyribonuclease I (EC 3.1.4.5) at a concentration of 50 μg./ml.

Activity of glucose-6-phosphatase (EC 3.1.3.9). This was estimated by the method described by Swanson (1955).

Protein determination. The method of Lowry, Rosebrough, Farr & Randall

(1951) for insoluble proteins was used. Commercial trypsin after dialysis was used as standard.

Extinction was measured in an Unicam SP 500 spectrophotometer.

RESULTS

Purity of mitochondria. The mitochondria isolated from green and etiolated primary rye leaves were but little contaminated with microsomes. The activity of glucose-6-phosphatase was about 2.7% that found in the leaf homogenate, this value being similar to the results reported by Borst, Ruttenberg & Kroon (1967) for mitochondrial preparations from animal liver.

Practically the same amount of DNA was found in mitochondria assayed directly and after treatment with deoxyribonuclease, i.e. 2.3 μg./l mg. of mitochondrial ether powder (prepared according to Nieman & Poulsen, 1963, 1967). Thus, any possible involvement of extramitochondrial DNA in the incorporation of [^{14}C]precursors into mitochondrial RNA seems to be excluded.

Incorporation of [^{14}C]*precursors into RNA.* Intact mitochondria of etiolated rye leaves incorporated [2-^{14}C]uracil and [6-^{14}C]orotate into their RNA with an intensity exceeding 2.5 and 4-fold, respectively, that of incorporation in green leaves (Table 1). In both cases the incorporation of [2-^{14}C]uracil was higher and therefore this compound appeared to be more suitable for experiments.

The incorporation *in vitro* of [2-^{14}C]uracil into RNA by various cell organellae isolated from rye leaves is presented in Table 1. The mitochondria of etiolated leaves showed the highest rate of incorporation, whereas in etioplasts the incorporation was very small. On the other hand, the mitochondria of green leaves and chloroplasts

Table 1

Incorporation of [2-^{14}C]*uracil and* [6-^{14}C]*orotate into RNA by rye leaf organellae*

Subcellular fractions were incubated with radioactive compounds as described in Materials and Methods. RNA was extracted by the phenol method, estimated at 260 mμ, and radioactivity was determined.

Organellae	Incorporation of		
	[2-^{14}C]uracil		[6-^{14}C]orotate
	(counts/min./mg. of RNA)	relative spec. act.	(counts/min./mg. of RNA)
Mitochondria from etiolated leaves	3840	1.0	720
Mitochondria from green leaves	1550	0.4	190
Chloroplasts	1580	0.4	—
Etioplasts	610	0.15	—
Microsomes from green leaves	110	0.03	—

were equally active, the rates of incorporation being about half that found for mitochondria of etiolated leaves. Microsomal RNA, both from green and etiolated leaves, showed the lowest, practically negligible, specific activities. This indicates that cytoplasmic ribosomes did not contribute to the activities found in mitochondrial RNA, even though some minor impurities were present in our mitochondrial preparations.

RNA turnover at submitochondrial level. The radioactivity of RNA isolated from mitochondrial subfractions after labelling of intact or disintegrated mitochondria, and after labelling of the isolated subfractions, is shown in Table 2. Irrespective whether intact or disintegrated mitochondria were incubated with labelled uracil, the same distribution of specific activities between the subfractions was obtained. The highest activity was found in the 100 000 *g* pellet, whereas the values for the 20 000 *g* pellet and the supernatant were much smaller. These results indicate that disintegration of mitochondria does not abolish their ability to incorporate the precursors, nor does it alter the characteristics of this incorporation.

Quite different results were obtained when the isolated mitochondrial subfractions were separately incubated with [^{14}C]uracil. Under these conditions only RNA of the supernatant was highly labelled and the RNA's isolated from the other two subfractions showed practically no activity.

RNA isolated from the 20 000 *g* pellet of mitochondria which had been incubated with [2-^{14}C]uracil and then disintegrated, was separated on MAK column into two fractions. The first one was eluted at 0.7 M-NaCl concentration, and the second at 1.3 M; they represented, respectively, the low-molecular-weight and the high-molecular-weight RNA fractions (Sueoka & Cheng, 1962). The activity of the first

Table 2

Distribution of radioactivity in mitochondrial subfractions after incorporation of [2-^{14}C]*uracil into RNA by intact and disintegrated mitochondria and by isolated mitochondrial subfractions of rye leaves*

The following preparations were incubated for 1 hr. with [2-^{14}C]uracil: intact and disintegrated mitochondria from etiolated leaves, and mitochondrial subfractions from green leaves. The mitochondria were fractionated as described in Materials and Methods, ether-dried powders prepared according to Nieman & Poulsen (1963), then RNA was extracted with cold 1 N-perchloric acid and estimated by the orcinol method.

Mitochondrial subfraction	Incorporation by		
	intact mitochondria	disintegrated mitochondria	isolated subfractions
	(counts/min./mg. of RNA)		
20 000 *g* pellet	800	1760	165
100 000 *g* pellet	3375	4800	175
100 000 *g* supernatant	860	2120	5500

fraction was always higher than that of the second one (Table 3). The ratio of specific activities of the two forms of RNA differed from one experiment to another over a range of 1.1 - 1.8.

Labelling of mitochondrial protein. The incorporation of [^{14}C]leucine into protein of mitochondria or mitochondrial subfractions isolated from green or etiolated leaves, was quite insignificant (Table 4). This very small protein biosynthesis suggests that in the experiments on RNA synthesis the contribution of bacterial contaminations was negligible.

Table 3

Specific activities of RNA's isolated from the 20 000 *g pellet subfraction of green rye leaf mitochondria and separated on MAK column*

Isolated mitochondria were incubated with [2-^{14}C]uracil as described in Materials and Methods, then disintegrated by sonic treatment, the 20 000 *g* pellet collected and RNA isolated by the phenol method. About 2 mg. of RNA in 0.05 M-phosphate buffer, pH 6.7, was applied to the column and stepwise elution was carried out with NaCl solutions in the same buffer. The fractions were separately pooled, dialysed against 0.01 M-phosphate buffer, pH 6.7, and evaporated to a small volume. RNA was estimated by E_{260} measurements.

Fraction eluted with NaCl conc. (M)	Specific activity (counts/min./mg. of RNA)
0.7	1530
1.3	865

Table 4

L-[U-^{14}C]*Leucine incorporation into proteins by mitochondria of rye leaves*

Isolation procedures for mitochondria and mitochondrial subfractions were as described in Materials and Methods. Mitochondria were incubated with L-[U-^{14}C]leucine as described under *Protein labelling*, then mitochondrial subfractions were isolated.

Expt. no.	Preparation in which, after incubation, radioactivity and protein were estimated	Incorporation (counts/min./mg. of protein)
1	Whole mitochondria from green leaves	65
	Whole mitochondria from etiolated leaves	55
2	Mitochondrial subfractions from green leaves:	
	20 000 *g* pellet	40
	100 000 *g* pellet	0
	100 000 *g* supernatant	60

DISCUSSION

The incorporation of nucleotide precursors into RNA is considered to represent RNA turnover. Recently, South & Mahler (1968) observed a correspondence of radioactivity profiles for RNA of yeast mitochondria labelled *in vitro* either with [^{3}H]ribonucleoside triphosphate as substrate for DNA-dependent RNA polymerase (EC 2.7.7.6) or with [^{14}C]uracil.

In the present work it was found that mitochondria derived both from green and etiolated rye leaves incorporated labelled uracil *in vitro*. Incorporation appeared to be higher in the latter case. The mitochondria of green plants, similarly as the chloroplasts, had a lower RNA turnover, whereas in cytoplasmic ribosomes the labelling of RNA was negligible.

Recently we observed (Rytel *et al.*, 1969b) that about 80% of the mitochondrial RNA was present in a heavy subfraction sedimented at 20 000 *g*. In the present work, the highest RNA turnover was found in the 100 000 *g* pellet, both when intact and disintegrated mitochondria were incubated with [^{14}C]uracil. These results indicate that RNA metabolism is not equal in all regions of the mitochondrion, being the highest in the 100 000 *g* pellet, although the other subfractions also participate in this process. On the other hand, when isolated mitochondrial subfractions were separately incubated with [^{14}C]uracil, only RNA from the 100 000 *g* supernatant was labelled. This may indicate that RNA polymerase was released into the supernatant during separation of mitochondrial subfractions.

During the last few years, attempts have been made to separate various mitochondrial subfractions defined in morphological and enzymic terms. Among others, Sottocasa, Kuylenstierna, Ernster & Bergstand (1967) and Schnaitman & Greenawalt (1968) succeeded in separating inner and outer membranes from rat liver mitochondria. To disrupt mitochondria, the latter authors used digitonine; a heavy subfraction consisting of the inner membranes and matrix was sedimented at 12 000 *g*, and, from the resultant supernatant, outer membranes were collected by centrifugation at 144 000 *g* for 1 hr.

Although our experimental conditions of both disintegration and subfractionation of mitochondria differed from those used by Schnaitman & Greenawalt (1968), it may be assumed that our 20 000 *g* pellet contained inner membranes and matrix, while the 100 000 *g* pellet consisted of outer membranes and mitochondrial "ribosomes". The 100 000 *g* supernatant seems to correspond to the soluble fraction described for rat liver mitochondria by Schnaitman & Greenawalt (1968). Thus, it is quite probable that RNA biosynthesis within the mitochondrion is compartmentized. Recently, Suyama & Eyer (1968) demonstrated that submitochondrial fractions of Tetrahymena obtained by differential centrifugation, showed different rates of RNA synthesis.

Differences in specific activity were observed also between the low- and high-molecular-weight forms of RNA from the 20 000 *g* pellet, separated on MAK column. Thus, it appears that not only RNA from the individual mitochondrial

subfractions, but also two forms of RNA differing in polymerization degree, may show different turnover rates.

So far, the data concerning the semiautonomous protein biosynthesis within the mitochondrion are conflicting (see ref. Work, Coote & Ashwell, 1968). Our results indicate that amino acid incorporation abilities of rye leaf mitochondria are negligible, at least under our experimental conditions.

Thus, the question arises what is the role of mitochondrial RNA. We agree with the assumption formulated recently by Davey, Yu & Linnane (1969) that mitochondrial RNA (messenger RNA), synthesized within the mitochondrion, is transported to the cytoplasm and there utilized in translation processes in cooperation with cytoplasmic ribosomes.

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BIOSYNTEZA KWASU RYBONUKLEINOWEGO W MITOCHONDRIACH LIŚCI ŻYTA (*SECALE CEREALE*) *IN VITRO*

Streszczenie

1. Badano *in vitro* procesy włączania [2-^{14}C]uracylu do RNA mitochondriów (nienaruszonych i rozbitych) liści żyta oraz rozmieszczenie znakowania w RNA występującym w podfrakcjach mitochondrialnych uzyskanych przez frakcjonowane wirowanie rozbitych mitochondriów.

2. W doświadczeniach, w których inkubowano z [2-^{14}C]uracylem mitochondria nienaruszone lub rozbite, najwyższą aktywność stwierdzano w RNA występującym w osadzie opadającym przy 100 000 *g*, natomiast gdy prowadzono inkubację izolowanych podfrakcji mitochondrialnych, jedynie RNA pozostający w supernatancie po 100 000 *g* był wysoko znakowany.

3. Uzyskane wyniki wskazują, że nienaruszona struktura mitochondriów nie jest niezbędna dla włączania przez nie uracylu do RNA.

4. Włączanie L-[U-^{14}C]leucyny do białek mitochondrialnych było nieznaczne.

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WIESŁAWA ŁYSIAK, ALEKSANDRA MUZALEWSKA and S. ANGIELSKI

BRANCHED-CHAIN AMINOTRANSFERASE IN BRAIN TISSUE

Laboratory of Clinical Biochemistry, Medical School, ul. Dębinki 7, Gdańsk 6, Poland

1. The activity of branched-chain aminotransferase was determined by enzymic assay of α-oxoglutarate utilization. 2. In homogenates of human and rat tissues, the highest enzyme activity was found in heart and brain, lower activity in kidney and only traces in liver. 3. Post-mitochondrial supernatant of rat brain contained 85% of the aminotransferase activity, whereas the mitochondrial fraction 10%. 4. The enzyme from pig brain was purified 100-fold using ammonium sulphate fractionation, DEAE-cellulose chromatography and Sephadex G-100 gel filtration.

The first step in the metabolism of branched-chain amino acids is the transamination with α-oxoglutarate. As a result of this reaction, α-oxoisocaproate is formed from leucine, α-oxoisovalerate from valine, and α-oxo-β-methylvalerate from isoleucine (Cammarata & Cohen, 1950; Awapara & Seale, 1952; Roswell, 1956a,b). Ichihara & Koyama (1966) and Taylor & Jenkins (1966) isolated from pig heart muscle an aminotransferase specific for all three amino acids. The distribution of the enzyme activity was examined in the liver, kidney, heart and skeletal muscles (Ichihara & Koyama, 1966) but not in brain tissue.

In branched-chain ketoaminoaciduria, a disease due to an inborn error in metabolism, the main symptoms are neurological damage and mental defect (Menkes, Hurst & Craig, 1954). The metabolic error consists in impairment of oxidative decarboxylation of branched-chain oxoacids (Dancis, Hutzler & Levitz, 1963; Snyderman, Norton, Roitman & Holt, 1964). Therefore the metabolism of branched-chain amino acids in the brain seems of special interest.

The aim of the present work was to compare the activity of branched-chain aminotransferase in different tissues of man and rat, attention being paid to brain tissue. Moreover, the enzyme has been isolated from pig brain and some of its properties are reported.

MATERIALS AND METHODS

Reagents. L-Amino acids were from Reanal (Budapest, Hungary), Sephadex G-100 from Pharmacia (Uppsala, Sweden), DEAE-cellulose from Sigma Co. (St. Louis, Mo., U.S.A.), 2-mercaptoethanol, pyridoxal phosphate and α-oxoisocaproic

acid from Fluka A. G. (Buchs S. G., Switzerland); the latter was converted to sodium salt according to Snell (1953); NADH, glutamate dehydrogenase (EC 1.4.1.2) and lactate dehydrogenase (EC 1.1.1.28) were from C. F. Boehringer & Soehne GmbH (Mannheim, German Federal Republic), tris from British Drug Houses (Poole, Dorset, England), α-oxoglutaric acid from Mann Research Lab. Inc. (New York, U.S.A.); maleic acid was a U.S.S.R. product, and bovine blood serum albumin was from Armour Pharmaceutical Comp. (Kankakee, Ill., U.S.A.). Other reagents were from P.O.Ch. (Gliwice, Poland).

Tissue homogenates. Human tissues were obtained from the Department of Forensic Medicine of the Medical School in Gdańsk thanks to the courtesy of Prof. Dr. S. Raszeja; tissue samples were taken within 2 - 5 hr. after death of persons who had been killed in traffic accidents. Wistar rats weighing 250 - 300 g. were stunned, killed by decapitation, and the tissues isolated.

Human and rat tissues were immersed in cold solution *A* consisting of 50 mM-sodium maleate (pH 8.0), 1 mM-EDTA, 1 mM-2-mercaptoethanol and 4 μM-pyridoxal phosphate. The cooled tissues were cut with scissors and homogenized with 2.5 vol. of solution *A* in a glass Potter-Elvehjem homogenizer provided with a teflon pestle at 600 rev./min. for 2 min. at 0°. The obtained homogenate was dialysed for 2.5 hr. at 4° against 20 vol. of solution *A*.

Pig brain used for enzyme isolation was collected in the municipal slaughterhouse immediately after killing of the animal, transported to the laboratory in ice, freed of *pia matter*, and either used directly or kept at -20° for a period not exceeding one month, no serious losses of enzyme activity being observed during this time. To prepare the homogenate, 600 g. of brain tissue (fresh or thawed) was homogenized with 5 vol. of solution *A* in a Warring blendor, twice for 1 min. at 5000 rev./min. and then for 1 min. at 11 000 rev./min. at 4°, and the enzyme purified as described in Results.

Preparation of subcellular fractions from rat brain. This was carried out according to Murthy & Rappoport (1963) in 0.25 M-sucrose - 1 mM-EDTA - 20 mM-tris-HCl buffer, pH 7.4. The fractions containing nuclei (1085 *g*) and mitochondria (10 000 *g*) were washed twice with the above sucrose solution. The mitochondrial pellet was suspended in the same volume of bidistilled water and left on ice for 20 min. Prior to enzyme determinations, the suspensions of nuclei and mitochondria, and the post-mitochondrial supernatant were dialysed for 2.5 hr. at 4° against 20 vol. of solution *A*.

Determination of branched-chain aminotransferase activity. The incubation mixture, final volume 0.5 ml., was composed of 40 mM-Na-pyrophosphate buffer, pH 8.6, the appropriate amino acid at 20 mM concentration, 8 mM-sodium α-oxoglutarate or pyruvate, 8 μM-pyridoxal phosphate, and enzyme preparation. To inhibit α-oxoglutarate oxidation, when liver homogenate was used, arsenite was added to a final concentration of 2 mM. When homogenates of brain, heart and kidney were used, it was not necessary to add arsenite because the homogenates contained maleate, the concentration of which in the incubation mixture amounted to 20 mM, and, as demonstrated by Angielski & Rogulski (1962), maleate inhibits

oxoglutarate oxidation in brain, heart and kidney, but not in liver. The incubation was carried out in an ultrathermostat at 37° for 10 min. The reaction was started by the addition of homogenate or enzyme preparation, and stopped with 0.5 ml. of 3.4% $HClO_4$. The control samples contained no amino acids. From the supernatant, a 25 - 100 µl. sample was taken for enzymic determination of the remaining α-oxoglutarate (Bergmeyer & Bernt, 1965) or pyruvate (Rosenberg & Rush, 1966). The sample was adjusted to 0.5 ml. with 0.1 M-K-phosphate buffer, pH 7.4. This amount of the buffer was sufficient to neutralize the sample, and no $KClO_4$ sediment was formed. To the sample was added 2.5 ml. of the same buffer containing 0.3 µmole of NADH, and, for α-oxoglutarate determination, 0.5 m-mole of ammonium sulphate and 10 µg. of glutamate dehydrogenase, or, for pyruvate determination, 50 µg. of lactate dehydrogenase. This mixture was left at room temperature and after 30 min. the decrease in extinction at 340 mµ was measured.

Protein was assayed by the biuret method according to Gornall *et al.* as described by Layne (1957), with bovine serum albumin as standard. The amount of protein in eluates from Sephadex G-100 column was also determined spectrophotometrically at 260 and 280 mµ according to Warburg (Layne, 1957).

RESULTS

In preliminary experiments carried out to choose the appropriate conditions for the assay of branched-chain aminotransferase activity in brain homogenate, the reaction rate was found to be linear with protein concentration from 0.5 to at least 4 mg., and linear with time of incubation to at least 30 min.; maximum activity was obtained at 7 mM-α-oxoglutarate concentration (Fig. 1).

The elaborated procedure was compared with the method applied by Ichihara & Koyama (1966) based on selective extraction of 2,4-dinitrophenylhydrazones of α-oxoglutarate and α-oxoisocaproate. The results obtained by the two methods with the same sample did not differ by more than 10%. The presented procedure is, however, simpler and less time-consuming.

The effect of pH on the aminotransferase activity in rat brain is shown in Fig. 2. The appropriate pH values were obtained by carrying out both the homogenization and the transamination reaction in the following buffers: maleate, pH 6.0; tris-HCl, pH 7.0, 7.4 and 8.0; and pyrophosphate, pH 8.6 and 9.1. Previously it was proved that the enzyme activity was not dependent on the kind of buffer used.

The optimum pH for the aminotransferase activity was found to be at about 8.6. The reverse reaction, in which 20 mM-L-glutamate and 8.0 mM-α-oxoisocaproate were used as substrates, showed the same pH optimum but the activity was by 40% lower.

Under the conditions studied, in rat brain no leucine - pyruvate aminotransferase activity was detected.

The activity of branched-chain aminotransferase in human and rat tissues. The activity in homogenates of livers, kidneys, hearts and brain is shown in Table 1;

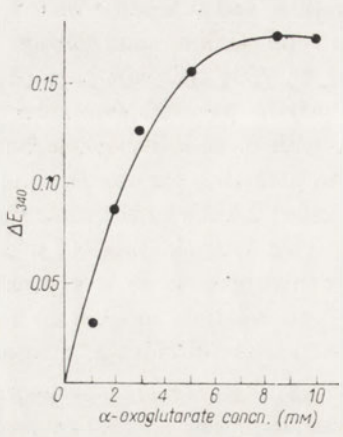

Fig. 1

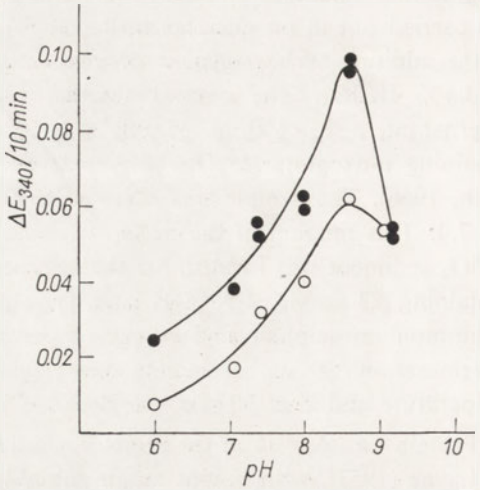

Fig. 2

Fig. 1. The effect of α-oxoglutarate concentration on the activity of leucine - α-oxoglutarate aminotransferase in rat brain homogenate. Conditions of the assay are described in Methods.

Fig. 2. The effect of pH on the activity of branched-chain aminotransferase in rat brain homogenate. Substrates: (●), 20 mM-Leucine and 8 mM-α-oxoglutarate; (○), 20 mM-glutamate and 8 mM-α-oxoisocaproate. Conditions of the assays are described in Methods.

the highest activity was observed in human heart, rat heart and rat brain. In kidney, the activity was 4 - 6 times lower in rat and twofold lower in man. In livers, both of man and rat, only traces of enzyme activity were found.

In rat tissues, the aminotransferase showed the highest activity toward valine as substrate, and a smaller one toward leucine and isoleucine. The ratios of activity for leucine : valine, and leucine : isoleucine were in all tissues almost the same. On the contrary, the ratio of activity for leucine : aspartate differed greatly.

With human tissues only two experiments were performed, therefore it was not possible to conclude whether there is a species-dependent difference in the activity of the enzyme toward the three amino acids.

Subcellular distribution of the aminotransferase in rat brain. Nearly the total activity was found in the post-mitochondrial fraction (Table 2), and only a small amount in mitochondria, which, however, showed a rather high specific activity. The low activity found in the nuclear fraction could be due to the presence of some intact cells and to incomplete removal of cytoplasm during washing.

Purification of branched-chain aminotransferase from pig brain. Brain homogenate, freshly prepared as described in Methods, was kept with constant stirring for 30 min. at 4°, then for 10 min. at 45° (on a water bath) and cooled (30 - 60 min.) in a mixture of ice, water and NaCl. All further steps of the procedure were carried out at 4°. The preparation was centrifuged for 30 min. at 15 000 *g*, the sediment was

Table 1

Branched-chain aminotransferase activity in rat and human tissue homogenates

The composition of the reaction mixture is given in Materials and Methods; α-oxoglutarate was used as amino group acceptor. Mean values from the number of experiments indicated in parentheses, ±S.D. are given.

Tissue	Substrate				Ratios of activity		
	Leu	Val	Ile	Asp	Leu/Val	Leu/Ile	Leu/Asp
	Activity (nmoles/mg. protein/10 min.)						
Rat (8) Liver	<1	<1	<1	66±8	—	—	—
Kidney	11±5	18±7	12±5	60±5	0.61	0.92	0.18
Heart	61±5	79±10	71±7	74±10	0.77	0.86	0.82
Brain	47±10	64±15	53±10	106±20	0.73	0.89	0.44
Man (2) Liver	<1	<1	<1	95	—	—	0.01
Kidney	29	35	32	135	0.83	0.91	0.21
Heart	68	65	73	164	1.04	0.93	0.40

Table 2

Subcellular distribution of branched-chain aminotransferase activity in rat brain

The composition of the reaction mixture is given in Materials and Methods; leucine and α-oxoglutarate were used as substrates. The activity is expressed in nmoles of α-oxoglutarate utilized/10 min.

Fraction	Protein (mg./ml.)	Total protein (mg.)	Activity		Relative activity (%)
			per 1 mg. protein	total	
Homogenate	8.5	297	85	25 000	100
Nuclei	19.0	152	10	1 520	6
Mitochondria	5.0	20	97	1 940	8
Post-mitochondrial supernatant	1.7	136	156	21 500	85

discarded and the supernatant was treated with solid ammonium sulphate. The precipitate at 0.4 - 0.7 saturation was collected by centrifugation, the sediment suspended in 150 ml. of a solution containing: 5 mM-sodium maleate, pH 8.0, 1 mM-2-mercaptoethanol, 1 mM-EDTA and 4 μM-pyridoxal phosphate (solution *B*), and dialysed overnight against 20 volumes of the same solution. The dialysis residue was centrifuged for 30 min. at 15 000 *g*, and to the supernatant, solid ammonium sulphate was added to 0.6 saturation. The precipitate was suspended in about 30 ml. of solution *B* and dialysed as above for 2 hr.

The dialysed clear enzyme solution was applied to a DEAE-cellulose column and the protein was eluted with a continuous KCl concentration gradient obtained from

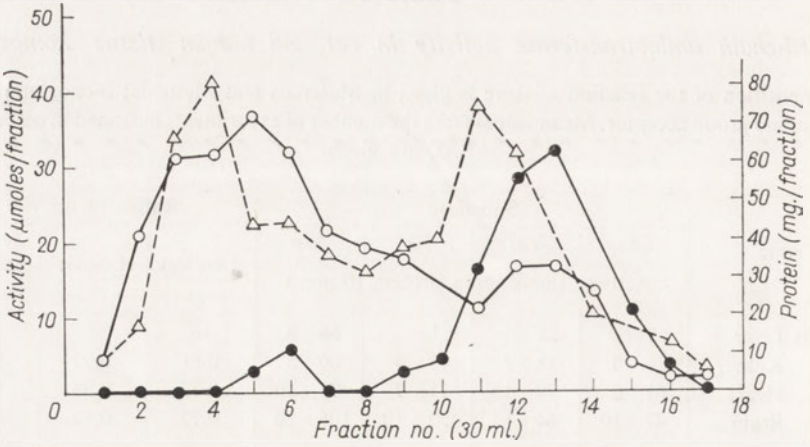

Fig. 3. DEAE-cellulose column chromatography of the aminotransferase preparation from pig brain (Table 3, step 4). DEAE-cellulose was washed with 0.5 N-NaOH, absolute ethanol and water (Peterson & Sober, 1957), then 25 g. was introduced into a column 2.5 cm. in diameter, and equilibrated for 48 hr. with solution *B*. Protein was eluted with a continuous KCl concentration gradient and activity determined. (●), Activity of leucine - α-oxoglutarate aminotransferase; (○), activity of aspartate - α-oxoglutarate aminotransferase; (△), protein.

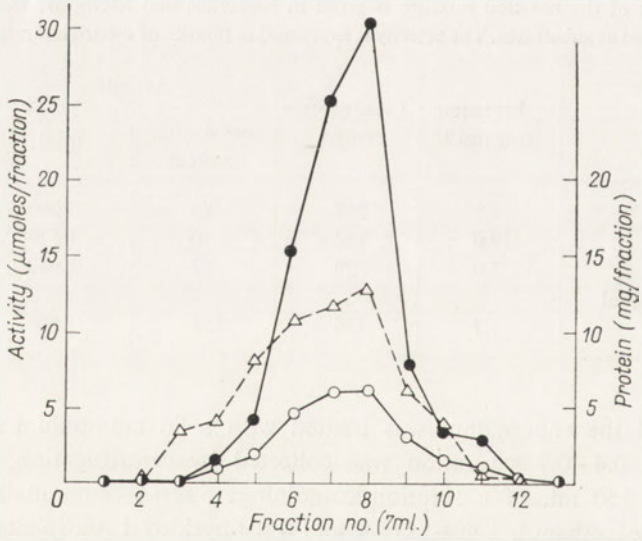

Fig. 4. Gel filtration on Sephadex G-100 of branched-chain aminotransferase from pig brain. The Sephadex, 2.5 - 3 g., was suspended in solution *B* and allowed to swell for 48 hr. at 4°, then it was introduced to a column 1.6 cm. in diameter, and equilibrated for 24 hr. with the same solution. The pooled fractions nos. 11 - 15 obtained after DEAE-cellulose chromatography were applied to the column and the enzyme was eluted with the KCl concentration gradient. (●), Activity of leucine - α-oxoglutarate aminotransferase; (○), activity of aspartate - α-oxoglutarate aminotransferase; (△), protein.

300 ml. of solution *B* in one vessel and 300 ml. of 0.5 N-KCl in solution *B* in another vessel (Fig. 3).

The fractions nos. 11 - 15 showing the highest enzyme activity were pooled and solid ammonium sulphate was added to 0.6 saturation. The sediment was suspended in solution *B* and dialysed overnight as described above, then applied to a Sephadex G-100 column, and the enzyme was eluted with the KCl concentration gradient as used for DEAE-cellulose column chromatography (Fig. 4).

In four series of enzyme purification, the yield of the enzyme was 5 - 20%. In comparison with the homogenate, the specific activity increased 50 - 100 times. One purification procedure is presented in Table 3.

Table 3

Purification of branched-chain aminotransferase from pig brain

The composition of the reaction mixture is given in Materials and Methods; leucine and α-oxoglutarate were used as substrates. The activity is expressed in nmoles of α-oxoglutarate utilized/10 min.

Step of purification	Volume (ml.)	Protein (mg./ml.)	Activity		Yield (%)	Purification factor
			per 1 mg. protein	total		
1. Homogenate	3570	8.25	64	1 807 000	100	
2. Heating at 45°	2680	3.80	125	1 262 000	71	2
3. Ppt. at 0.4 - 0.7 $(NH_4)_2SO_4$ sat.	128	24.10	380	1 183 000	66	6
4. Ppt. at 0.6 $(NH_4)_2SO_4$ sat.	21	54.00	665	735 000	40	10
5. DEAE-cellulose	5.5	34.00	2600	485 000	27	41
6. Sephadex G-100	14.2	2.90	7900	325 000	18	115

Table 4

Specificity of the purified branched-chain aminotransferase preparation

The composition of the reaction mixture is given in Materials and Methods. α-Oxoglutarate was used as amino group acceptor.

Substrate	Activity (nmoles of α-oxoglutarate utilized/mg. protein/10 min.)	Relative activity (%)
L-Leucine	7900	100
L-Isoleucine	9400	119
L-Valine	7750	98
L-Aspartate	1500	19
L-Phenylalanine	545	6.8
L-Alanine	trace	< 1
β-Alanine	trace	< 1
Glycine	trace	< 1

Table 5

Stability of the purified preparation of branched-chain aminotransferase

The composition of the reaction mixture is given in Materials and Methods. Leucine and α-oxoglutarate were used as substrates.

Days of storage at 4°	Activity (%)
1	100
2	100
6	75
10	67
14	22

Substrate specificity of the purified brain enzyme. For this assay, fraction no. 8 from Sephadex G-100 column was used. The preparation showed the highest activity toward isoleucine, and somewhat lower activity toward leucine and valine (Table 4); the preparation was contaminated with aspartate - α-oxoglutarate aminotransferase, the activity of which corresponded to 20% of that toward leucine. The remaining amino acids studied, L-phenylalanine, L-alanine, β-alanine and glycine, showed but traces of transamination with α-oxoglutarate.

Stability of the enzyme. The purified preparation kept in solution *B* at 4°, showed after a week 70%, and after two weeks 20%, of the initial activity (Table 5).

DISCUSSION

The high activity of branched-chain aminotransferase observed in rat brain, is of special interest. Over 80% of the enzyme activity was found in the post-mitochondrial fraction, which seems to indicate that the transamination of leucine, valine and isoleucine occurs mainly in the cytoplasm whereas the oxidation of the formed α-oxoderivatives proceeds in mitochondria.

Among the tissues studied, the lowest activity of the aminotransferase was observed in liver, in agreement with the results of Ichihara & Koyama (1966). Thus, it appears that liver plays but a slight role in the first step of the metabolism of branched-chain amino acids. This conclusion is also supported by the studies of McManemy, Vang & Drapanes (1965) who found no increase in the content of branched-chain amino acids in blood plasma after hepatectomy.

Ichihara & Koyama (1966) and Taylor & Jenkins (1966) suggested that there is a single aminotransferase specific for all three branched-chain amino acids. The observed constant ratio of the activity toward leucine to valine, and leucine to isoleucine, in all studied tissues of a given species, are consistent with this view. On the other hand, the ratio of leucine to aspartate activity varied depending on the species and tissue tested, which indicates that the enzyme studied is different from

aspartate - α-oxoglutarate aminotransferase. Branched-chain aminotransferase isolated from pig brain was found to react with α-oxoglutarate, but not with pyruvate.

The enzyme isolated from pig brain, on DEAE-cellulose column chromatography showed two activity peaks, which could point to the presence of an isoenzyme, possibly of mitochondrial origin. This conclusion is based on the observation that the distribution of activity in the two chromatographic fractions is similar to the distribution of activity between the post-mitochondrial supernatant and the mitochondrial fraction.

Since the activity of branched-chain aminotransferase in particular tissues of rat and man is similar, and high activity of the enzyme has been found in rat brain, it seems reasonable to suppose that also in human brain its activity would be rather high. The blocking of oxidative decarboxylation of branched-chain amino acids occurring in branched-chain ketoaminoaciduria would result in accumulation of these compounds, mainly in those tissues which show the highest activity of branched-chain aminotransferase, i.e. brain tissue and heart muscle.

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AMINOTRANSFERAZA AMINOKWASÓW O ROZGAŁĘZIONYM ŁAŃCUCHU WĘGLOWYM W MÓZGU

Streszczenie

1. Opracowano metodę oznaczania aktywności enzymu opartą na enzymatycznym pomiarze ilości zużytego ketoglutaranu.

2. Aktywność enzymu, badana w tkankach człowieka i szczura, była najwyższa w sercu i mózgu, niższa w nerce, a tylko ślady aktywności stwierdzono w wątrobie.

3. W mózgu szczura prawie cała aktywność (85%) była zlokalizowana we frakcji pomitochondrialnej, a tylko 10% znaleziono we frakcji mitochondrialnej.

4. Enzym z mózgu wieprza oczyszczono 100-krotnie stosując frakcjonowanie siarczanem amonowym, chromatografię na kolumnie DEAE-celulozy oraz filtrację na żelu Sephadex G-100.

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WIESŁAWA ŁYSIAK, J. STĘPIŃSKI and S. ANGIELSKI

INHIBITION OF α-OXOGLUTARATE AND PYRUVATE OXIDATION BY α-OXODERIVATIVES OF LEUCINE AND VALINE IN RAT TISSUES

Laboratory of Clinical Biochemistry, Medical School, ul. Dębinki 7, Gdańsk 6, Poland

1. α-Oxoisocaproate and α-oxoisovalerate inhibited the oxidation of α-oxoglutarate and pyruvate in mitochondria. The inhibition was the greatest in liver, and smaller in kidney, heart and brain. 2. In rat heart mitochondria disintegrated by sonic treatment, α-oxoisocaproate inhibited non-competitively the activity of α-oxoglutarate dehydrogenase. 3. The inhibitors had no effect on oxidation of isocitrate and malate by liver mitochondria. 4. On this basis it is suggested that the observed inhibition of α-oxoglutarate and pyruvate oxidation by branched-chain oxoacids is related mainly to the similarity in their metabolism, and thus to the competition for free coenzyme A and probably to the inhibitory effect of acyl derivatives of coenzyme A on α-oxoglutarate and pyruvate dehydrogenases.

Branched-chain ketoaminoaciduria, known as maple syrup urine disease (Menkes, Hurst & Craig, 1954), is an inborn error in metabolism, in which the primary metabolic lesion concerns the oxidative decarboxylation of α-oxoacids derived from branched-chain amino acids, leucine, isoleucine and valine (Dancis, Levitz, Miller & Westall, 1959; Dancis, Hutzler & Levitz, 1965). However, it has been recently demonstrated that the metabolism of α-oxoisovalerate formed from valine, is not impaired (Snyderman, Norton, Roitman & Holt, 1964; Bowden & Connelly, 1968). In ketoaminoaciduria, branched-chain α-oxoacids and amino acids are accumulated in tissues and in blood, which in turn leads to their increased excretion in urine. Patric (1961) and then Woody & Harris (1965) observed that the urine of children with branched-chain ketoaminoaciduria contained also increased amounts of α-oxoglutarate. The increased excretion of this compound could be due to the inhibitory action of branched-chain amino acids or their oxoderivatives on α-oxoglutarate reabsorption from renal tubules, or to the inhibition of the activity of α-oxoglutarate and pyruvate dehydrogenases. The latter possibility seems to be more probable in view of the similarity in the mechanism of oxidative decarboxylation of the four compounds.

Swaiman & Milstein (1961) observed that leucine, valine and isoleucine did not inhibit *in vitro* the oxidation of glucose by rabbit brain slices. On the other hand Tashian (1961) demonstrated a slight inhibition of glutamate decarboxylation by

branched-chain α-oxoacids. Recently Dreyfus & Prensky (1967) have shown that α-oxoisocaproate, at relatively high (15 mM) concentration, inhibits by 50% pyruvate decarboxylation by rat liver homogenates.

The aim of the present work was to study the effect of α-oxoisocaproate and α-oxoisovalerate on the oxidation of α-oxoglutarate and pyruvate in mitochondria isolated from rat tissues. Both compounds studied appeared to be inhibitors, the liver mitochondria being most sensitive to the inhibition. α-Oxoisocaproate inhibited the oxidation of pyruvate, but not of α-oxoglutarate, by brain mitochondria.

MATERIALS AND METHODS

Reagents. Sodium pyruvate, $NADP^+$, and hexokinase, type III, were purchased from Sigma Chem. Co. (St. Louis, Mo., U.S.A.); ATP, NADH, ADP, lactate dehydrogenase and glutamate dehydrogenase from C. F. Boehringer & Soehne GmbH (Mannheim, German Federal Republic); L-amino acids from Reanal (Budapest, Hungary); sodium DL-isocitrate and isocitrate dehydrogenase from Koch-Light Lab. Ltd. (Colnbrook, Bucks, England); sodium α-oxoisovalerate from Mann Research Lab. Inc. (New York, U.S.A.); α-oxoisocaproic acid (A. G. Fluka, Buchs S. G., Switzerland) was converted to the sodium salt according to Snell (1953). α-Oxoglutaric acid (P.O.Ch., Gliwice, Poland) was recrystallized as monosodium salt according to Krebs, Eggelston & D'Alessandro (1961). Other reagents were from P.O.Ch. (Gliwice, Poland).

Preparation of homogenates and mitochondria. Wistar rats weighing 200 - 300 g. were stunned and killed by decapitation. The tissues were immediately isolated and immersed in cold 0.25 M-sucrose - 1 mM-EDTA solution. The brain was homogenized with 2 vol., and the other tissues with 4 vol. of the isotonic sucrose solution, in a glass Potter-Elvehjem homogenizer provided with a teflon pestle, at 600 rev/min. for 2 min. at 0°.

Mitochondria of liver and kidney were prepared according to Hogeboom (1957), those of heart according to Plaut & Plaut (1952) and of brain according to Brody & Bain (1952). The obtained mitochondria were washed twice with 0.25 M-sucrose, and suspended in isotonic sucrose containing 1 mM-EDTA.

Heart mitochondria were used either directly or after disintegration by ultrasonic oscillations at 20 kHz for 30 sec. at 4°. After centrifuging for 10 min. at 12 000 *g*, the sediment was discarded and the supernatant dialysed against 100 vol. of 10 mM-potassium phosphate buffer, pH 7.4, at 4°.

Conditions of incubation. The effect of α-oxoisocaproate and α-oxoisovalerate on oxidation of α-oxoglutarate, pyruvate, L-malate and DL-isocitrate was studied in a medium containing in a final volume of 2 ml.: 40 μmoles of potassium phosphate buffer, pH 7.4, 80 μmoles of KCl, 1.25 μmoles of ADP, 10 μmoles of magnesium sulphate, 60 μg. of hexokinase, 120 μmoles of glucose and 0.2 ml. of the mitochondrial suspension, containing 3 - 5 mg. of protein. The concentration of the medium was

brought to 270 - 300 m-osmolarity by adding appropriate amounts of 1 M-sucrose solution. α-Oxoglutarate, pyruvate and L-malate were added in amounts of 10μmoles, and DL-isocitrate of 20 μmoles. Moreover, when pyruvate or DL-isocitrate was used as substrate, 0.5 μmole of L-malate was added. The incubation was carried out in a Warburg apparatus in air, at 30° for 30 min. The reaction was stopped by adding 0.2 ml. of 30% $HClO_4$. The oxygen uptake was measured manometrically, and in the deproteinized supernatant the remaining substrate and P_i were determined.

The assays on α-oxoisocaproate oxidation were carried out in a medium containing in a final volume of 2 ml.: 100 μmoles of tris-HCl buffer, pH 7.4, 40 μmoles of KCl, 4 μmoles of α-oxoisocaproate and 0.5 ml. of tissue homogenate. The incubation was carried out in a Warburg apparatus, in air, at 37° for 40 min. The reaction was stopped by pouring 1 ml. of the incubation mixture into 7 ml. of 3% trichloroacetic acid, and the remaining substrate was determined.

The activity of α-oxoglutarate dehydrogenase (EC 1.2.4.2) was assayed in a medium containing in a volume of 1.5 ml.: 75 μmoles of potassium phosphate buffer, pH 7.4, 0.1 μmole of CoA, 7.5 μmoles of cysteine, 1 μmole of EDTA, 1.5 μmoles of $MgCl_2$, 1 μg. of antimycin, α-oxoglutarate, and the preparation from disintegrated heart mitochondria corresponding to 0.12 mg. of protein. After 3 min. of preincubation at 23°, the reaction was started by addition of 1.5 μmoles of NAD^+. The rate of NADH formation was measured at 340 nm at 15 sec. intervals.

Analytical methods. α-Oxoglutarate was assayed according to Bergmeyer & Bernt (1965), pyruvate according to Rosenberg & Rush (1966), isocitrate according to Stern (1957). Inorganic phosphate was determined by the method of Gomori (1953) and protein by the method of Gornall *et al.* as described by Layne (1957).

α-Oxoisocaproate was estimated in the following way: After incubation, to 1 ml. of the deproteinized solution, 1 ml. of 0.0125% 2, 4-dinitrophenylhydrazine solution in 2 N-HCl was added. The sample was left for 25 min. at room temperature, then 7 ml. of 1.5 N-NaOH was added, and after 5 min. the extinction at 440 nm in 1 cm. light-path cuvettes was measured. The calibration curve obtained under these conditions was linear up to 0.3 μmole of α-oxoisocaproate per sample; 0.10 μmole of α-oxoisocaproate gave an extinction of 0.175.

RESULTS

The effect on oxoglutarate oxidation. α-Oxoisocaproate, the derivative of leucine, inhibited the oxidation of oxoglutarate by mitochondria of liver, kidney and heart, whereas it had no effect, even at a concentration as high as 10 mM, on brain mitochondria (Table 1). The most sensitive to the inhibitory action were liver mitochondria. The observed inhibition of the disappearance of oxoglutarate was accompanied by the inhibition of uptake of oxygen and inorganic phosphorus.

α-Oxoisovalerate, the derivative of valine, was a more effective inhibitor of oxoglutarate oxidation; moreover, it inhibited the oxidation in brain mitochondria. The highest inhibition was again observed in liver.

Table 1

Inhibition of α-oxoglutarate oxidation by α-oxoisocaproate (KiC) and α-oxoisovalerate (KiV) by mitochondria of rat tissues

Composition of the incubation mixture is described in Methods. The utilization of oxoglutarate and inorganic phosphate is expressed as μmoles/10 mg. protein/30 min., and the uptake of oxygen in μg atoms/10 mg. protein/30 min. Mean values±S.D. are given; the number of experiments is given in parentheses.

Mitochondria from	Addition (mM)	Oxo-glutarate utilized	Inhi-bition (%)	O_2 uptake	Inhi-bition (%)	P_i uptake	Inhi-bition (%)
Liver	None,						
(6)	control	14±3.0		21±2.0		59±4.0	
	KiC 2.5	10±1.0	27	16±2.0	24	43±2.0	27
	KiC 10.0	6±1.0	57	9±1.0	57	24±3.0	59
	KiV 2.5	5±2.0	64	10±1.0	52	25±2.0	57
	KiV 10.0	4±1.0	71	8±1.0	71	21±2.0	61
	KiC 2.5						
	+KiV 2.5	6±1.0	57	11±2.0	48	29±2.0	51
Kidney	None,						
(4)	control	23±2.0		41±4.0		95±5.0	
	KiC 2.5	21±0.5	9	39±4.0	5	89±4.0	6
	KiC 10.0	14±1.0	39	24±3.0	41	52±5.0	45
	KiV 2.5	19±1.0	17	36±4.0	12	80±5.0	16
	KiV 10.0	11±1.0	52	20±3.0	51	45±5.0	44
	KiC 2.5						
	+KiV 2.5	18±1.5	21	35±3.0	15	83±4.0	12
Heart	None,						
(3)	control	20±4.0		31±2.0		52±3.0	
	KiC 2.5	17±1.0	15	28±3.0	10	43±3.0	17
	KiC 10.0	15±1.0	25	24±1.0	23	35±1.0	33
	KiV 2.5	16±1.0	20	22±2.0	29	40±3.0	23
	KiV 10.0	11±1.0	45	18±3.0	42	25±1.0	50
	KiC 2.5						
	+KiV 2.5	15±1.0	25	24±2.0	23	43±2.0	17
Brain	None,						
(5)	control	16±3.0		29±2.0		67±4.0	
	KiC 2.5	15±3.0	6	30±1.0	+4	68±3.0	+2
	KiC 10.0	17±2.0	+6	28±1.0	3	65±5.0	3
	KiV 2.5	13±2.0	19	24±1.0	17	53±1.0	21
	KiV 10.0	8±1.0	50	16±1.0	45	31±1.0	53
	KiC 2.5						
	+KiV 2.5	15±2.0	6	26±2.0	10	52±3.0	15

Simultaneous addition to the incubation mixture of the two oxoacids neither potentiated nor abolished the inhibition caused by one of them. As the effect was not additive, it is possible to assume that both inhibitors affected the same step of α-oxoglutarate metabolism.

The effect of concentration of the two inhibitors on oxoglutarate oxidation by liver mitochondria is shown in Fig. 1; 50% inhibition was observed at 6.8 mM-α-oxoisocaproate, and at 1.7 mM-α-oxoisovalerate concentration.

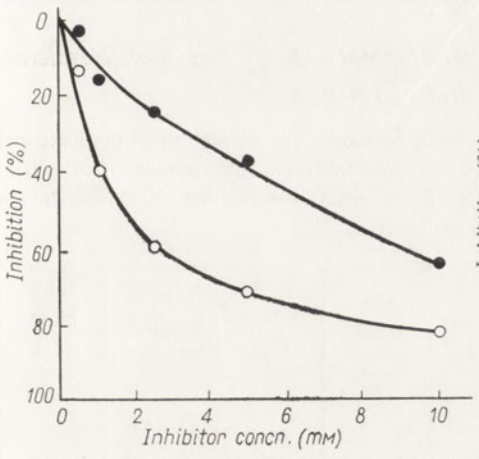

Fig. 1

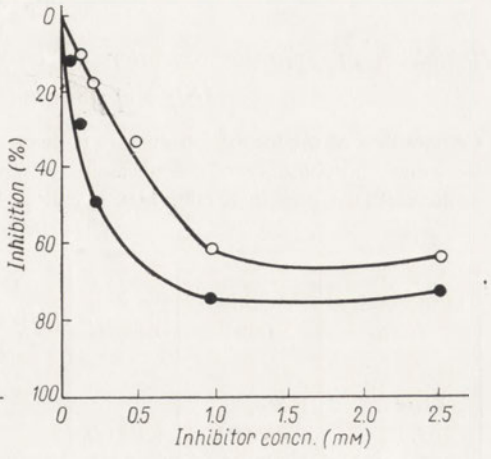

Fig. 2

Fig. 1. Inhibition of α-oxoglutarate oxidation in rat liver mitochondria by (●), α-oxoisocaproate and (○), α-oxoisovalerate. The incubation mixture was as described in Methods; after 30 min. the amount of the remaining substrate was determined.

Fig. 2. Inhibition of pyruvate oxidation in rat liver mitochondria by (●), α-oxoisocaproate and (○), α-oxoisovalerate. The incubation mixture was as described in Methods; after 30 min. the amount of the remaining substrate was determined.

Table 2

Oxidation of α-oxoisocaproate by homogenates of rat tissues

Mean values from 4 experiments are given ± S.D.

Homogenate from	α-Oxoisocaproate utilized (μmoles/g. of tissue/hr.)
Liver	4.7±0.8
Kidney	4.6±0.6
Heart	2.2+0.4
Brain	0.8±0.3

In Table 2 is presented the utilization of α-oxoisocaproate by tissue homogenates. From a comparison of these results with the inhibition of oxoglutarate oxidation it appears that there is some relation between the utilization of α-oxoisocaproate and its inhibitory effect. In liver and kidney, where the metabolism of α-oxoisocaproate

was the greatest, also the greatest inhibition of α-oxoglutarate oxidation was observed. On the other hand, in the brain homogenate the disappearance of α-oxoisocaproate was but slight and the inhibition of α-oxoglutarate oxidation was

Table 3

Inhibition of pyruvate oxidation by α-oxoisocaproate (KiC) and α-oxoisovalerate (KiV) in mitochondria of rat tissues

Composition of the incubation mixture is described in Methods. The utilization of pyruvate and inorganic phosphate is expressed as μmoles/10 mg. protein/30 min., the uptake of oxygen as μg atoms/10 mg. protein/30 min. Mean values±S.D. are given; the number of experiments is given in parentheses.

Mitochondria from	Addition (mM)	Pyruvate utilized	Inhibition (%)	O_2 uptake	Inhibition (%)	P_i uptake	Inhibiton (%)
Liver	None,						
(5)	control	8.5±1.0		14±2.0		34±3.0	
	KiC 2.5	2.6±0.5	69	8±1.0	44	9±2.0	73
	KiC 10.0	1.8±0.5	79	5±0.5	64	6±2.0	78
	KiV 2.5	3.6±0.5	58	9±1.0	37	15±2.0	56
	KiV 10.0	1.9±0.3	78	7±2.0	51	12±2.0	65
	KiC 2.5						
	+KiV 2.5	2.2±0.5	74	6±2.0	74	7±1.0	78
Kidney	None,						
(4)	control	8.2±0.6		26±3.0		66±5.0	
	KiC 2.5	6.6±0.6	20	27±4.0	+4	61±2.0	8
	KiC 10.0	5.1±0.6	38	23±6.0	8	52±5.0	21
	KiV 2.5	7.2±0.6	12	27±2.0	+4	66±6.0	0
	KiV 10.0	6.1±0.5	26	23±4.0	8	55±3.0	17
	KiC 2.5						
	+KiV 2.5	6.7±0.5	18	26±3.0	0	60±4.0	9
Heart	None,						
(3)	control	11.6±3.0		37±3.0		75±5.0	
	KiC 2.5	6.2±1.0	47	28±4.0	25	39±6.0	48
	KiC 10.0	4.4±1.0	62	17±2.0	54	25±6.0	66
	KiV 2.5	9.6±3.0	13	36±3.0	3	63±7.0	16
	KiV 10.0	6.6±2.0	43	19±4.0	49	35±6.0	53
	KiC 2.5						
	+KiV 2.5	8.9±2.0	16	33±3.0	11	58±5.0	23
Brain	None,						
(5)	control	12.7±0.5		41±2.0		89±5.0	
	KiC 2.5	6.3±0.5	50	21±2.0	49	34±5.0	62
	KiC 10.0	3.1±1.0	76	18±1.0	56	20±4.0	78
	KiV 2.5	13.1±1.5	+3	37±3.0	11	74±6.0	17
	KiV 10.0	8.5±1.5	33	30±2.0	28	51±5.0	43
	KiC 2.5						
	+KiV 2.5	7.9±1.0	38	24±2.0	41	46±5.0	48

not observed. This suggests that the inhibitory effect is related to a similarity in the metabolism of the two compounds.

The effect on pyruvate oxidation. The mitochondria studied oxidized pyruvate much less efficiently than α-oxoglutarate (Table 3), the greatest differences being found for kidney mitochondria in which the disappearance of pyruvate was about one-third of that observed for α-oxoglutarate.

α-Oxoisocaproate, which in brain mitochondria had no effect on oxidation of α-oxoglutarate, inhibited the oxidation of pyruvate. At 2.5 mM-α-oxoisocaproate concentration, 50% inhibition was observed, whereas α-oxoisovalerate at the same concentration had no effect. Similarly as in the case of oxoglutarate oxidation, both inhibitors had the greatest effect on liver mitochondria, and 2.5 mM-α-oxoisocaproate inhibited the oxidation of pyruvate by as much as 70%. However, the degree of inhibition was not proportional to inhibitor concentration (Fig. 2): 50% inhibition was attained at 0.2 mM-α-oxoisocaproate concentration, and at 0.65 mM-α-oxoisovalerate.

Kidney mitochondria were less sensitive to the action of either of the two inhibitors. Even at concentrations of 10 mM, the inhibition of pyruvate utilization was very small.

The effect on oxidation of L-*malate and* DL-*isocitrate by liver mitochondria.* Oxidation of L-malate (measured by oxygen uptake) was not inhibited by α-oxoisocaproate or α-oxoisovalerate (Table 4). At 10 mM concentration of either of these compounds, there was some inhibition of the disappearance of inorganic phosphate, but the difference was not statistically significant. Oxidation of isocitrate was not affected by the two oxoacids (Table 5).

The effect on the activity of α-oxoglutarate dehydrogenase. In disintegrated rat heart mitochondria, the activity of α-oxoglutarate dehydrogenase was inhibited by α-oxoisocaproate. The inhibition was of the non-competitive type (Fig. 3) as the inhibitor caused a decrease in V_{max} and did not affect the K_m value.

Table 4

Effect of α-oxoisocaproate (KiC) and α-oxoisovalerate (KiV) on oxygen uptake and P_i *disappearance in rat liver mitochondria, in the presence of malate as substrate*

Composition of the incubation mixture is described in Methods. The utilization of inorganic phosphate is expressed as μmoles/10 mg. protein/30 min., the uptake of oxygen as μg atoms/10 mg. protein/30 min. Mean values from 3 experiments ± S. D. are given.

Addition (mM)	O_2 uptake	P_i uptake	P/O ratio
None, control	11.8±3.0	31.6±6.0	2.7
KiC 2.5	15.0±2.0	33.2±5.0	2.2
KiC 10.0	11.8±2.0	23.4±4.0	2.0
KiV 2.5	14.6±2.0	32.2±2.0	2.2
KiV 10.0	10.8±2.0	22.4±3.0	2.0

Table 5

Effect of α-oxoisocaproate (KiC) and α-oxoisovalerate (KiV) on oxidation of DL-*isocitrate by rat liver mitochondria*

Composition of the incubation mixture is described in Methods. The utilization of isocitrate and inorganic phosphate is expressed as μmoles/mg. protein/30 min., the uptake of oxygen as μg atoms/10 mg. protein/30 min. Mean values from 2 experiments are given.

Addition (mM)	Isocitrate utilized	O_2 uptake	P_i uptake
None, control	16.8	21.9	58
KiC 2.5	16.6	22.6	61
KiC 10.0	16.6	24.4	57
KiV 2.5	16.6	27.7	63
KiV 10.0	16.6	23.2	53

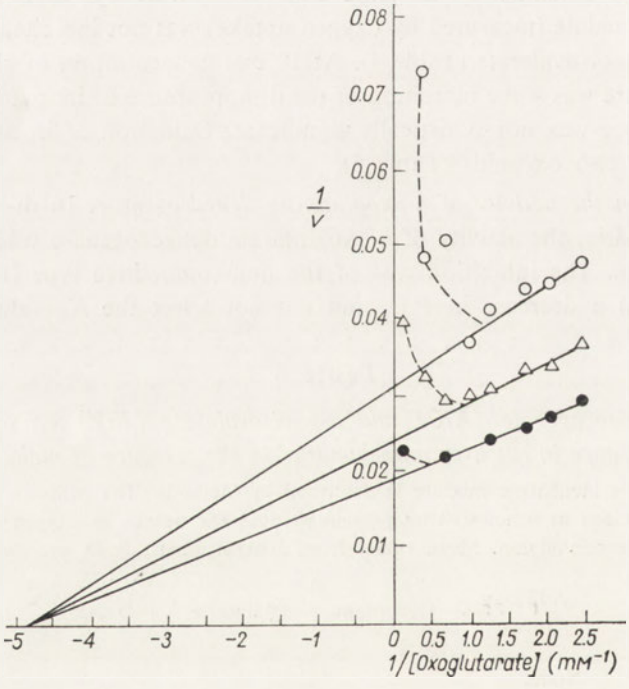

Fig. 3. Lineweaver-Burk plots for oxidation of α-oxoglutarate by disintegrated rat heart mitochondria (●), uninhibited; (△), in the presence of 1 mM-α-oxoisocaproate; (○), in the presence of 2 mM-α-oxoisocaproate. The incubation mixture was as described in Methods. Velocity, *v*, is expressed as ΔE_{340}/min./mg. protein. The activity of the disintegrated mitochondria amounted to 0.1 μmole NADH/mg. protein/min.

DISCUSSION

Inhibition of oxidation of α-oxoglutarate and pyruvate in mitochondria by α-oxoisocaproate and α-oxoisovalerate may be due to several factors. The first one may be the inhibition of the transport of the substrates into the mitochondrion; the second, blocking of the dehydrogenase systems either by the oxoacids themselves or by their metabolites; the third, competition for coenzymes participating in oxidative decarboxylation of α-oxoglutarate and pyruvate as well as of α-oxoisocaproate and α-oxoisovalerate.

In liver mitochondria, in which the greatest inhibition of oxoglutarate and pyruvate oxidation was found, the oxidation of DL-isocitrate, which undergoes decarboxylation without the participation of coenzyme A, was not inhibited, and there was no inhibition of L-malate oxidation. Thus it seems possible to assume that the mechanism of the inhibition of α-oxoglutarate and pyruvate oxidation does not consist either in the competition for the respiratory chain or in the uncoupling of oxidative phosphorylation.

It seems possible to exclude also the competition for penetration of the substrates into the mitochondrion as the main cause of inhibition. The results obtained with disintegrated mitochondria indicate that the inhibition of the activity of α-oxoglutarate dehydrogenase occurs also after removal of the mitochondrial membrane.

On the basis of the presented experiments it is possible to assume that the inhibition of α-oxoglutarate and pyruvate oxidation in the presence of α-oxoisocaproate and α-oxoisovalerate is related to the similarity of their metabolic pathways.

From the studies of Connelly, Danner & Bowden (1968) it appears that α-oxoisocaproate and α-oxoisovalerate, like α-oxoglutarate and pyruvate, undergo oxidative decarboxylation to isovaleryl-CoA, and isobutyryl-CoA, respectively. In the course of further reactions, acetoacetate and acetyl-CoA are formed from α-oxoisocaproate, and succinyl-CoA from α-oxoisovalerate. This may lead to a shortage of free coenzyme A, and the accumulating acyl derivatives of coenzyme A may cause an inhibition of the activity of α-oxoglutarate and pyruvate dehydrogenases. As demonstrated by Garland & Randle (1964), the activity of pyruvate dehydrogenase is inhibited by acetyl-CoA, and α-oxoglutarate dehydrogenase (Erfle & Sauer, 1969), also by succinyl-CoA.

The possibility of this mechanism being operative in the inhibition of α-oxoglutarate oxidation is supported by the relationship between the utilization of α-oxoisocaproate by different tissues and their sensitivity to inhibition. In liver, where α-oxoisocaproate utilization is very high, there is also the greatest inhibition of α-oxoglutarate oxidation, whereas in brain, in which no inhibition was observed, the α-oxoisocaproate is very poorly utilized.

The higher inhibition caused by α-oxoisovalerate may be due to the fact that succinyl-CoA is formed from this compound as well as from α-oxoglutarate, and the excess of succinyl-CoA inhibits the activity of α-oxoglutarate dehydrogenase.

In the case of pyruvate oxidation, α-oxoisocaproate was found to be a more potent inhibitor than α-oxoisovalerate. As α-oxoisocaproate, like pyruvate, is con-

verted to acetyl-CoA, this may lead to a relative shortage of oxaloacetate. Moreover, taking into account the structural similarity of α-oxoisocaproate and α-oxoisovalerate to pyruvate, direct action of these compounds on pyruvate dehydrogenase seems quite probable. May be this is why 50% inhibition of pyruvate oxidation was obtained with 0.2 mM-α-oxoisocaproate and 0.65 mM-α-oxoisovalerate, whereas for α-oxoglutarate oxidation the respective values were 6.8 and 1.7 mM.

The question arises whether the presented experiments may correspond to the conditions occurring in branched-chain ketoaminoaciduria.

The studies of Bowden & Connelly (1968) indicate that there are two enzymes catalysing the oxidative decarboxylation of oxoderivatives of branched-chain amino acids, one of them acting on oxoderivatives of leucine and isoleucine, and another acting on α-oxoisovalerate derived from valine. Moreover, these authors have demonstrated that α-oxoisocaproate inhibits competitively the activity of α-oxoisovalerate dehydrogenase, and α-oxoisovalerate the activity of α-oxoisocaproate dehydrogenase. According to the supposition of Menkes (1959), confirmed clinically by Snyderman *et al.* (1964), in branched-chain ketoaminoaciduria the metabolism of leucine, and possibly isoleucine is blocked, and the increase in the amount of valine and α-oxoisovalerate is due to the competitive inhibition by α-oxoisocaproate.

As demonstrated in the accompanying paper (Łysiak, Muzalewska & Angielski, 1970), the brain contains a highly active branched-chain aminotransferase and relatively high amounts of oxoderivatives are formed. In branched-chain ketoaminoaciduria, the impairment of α-oxoisocaproate dehydrogenase leads to the accumulation of α-oxoisocaproate, which in turn results in accumulation of α-oxoisovalerate. The accumulated α-oxoacids inhibit the oxidation of pyruvate and oxoglutarate. Thus it seems that the inhibition of pyruvate and α-oxoglutarate oxidation might be the main factor leading to brain damage and mental defect, observed as main symptoms of branched-chain ketoaminoaciduria.

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INHIBICJA SPALANIA α-OKSOGLUTARANU I PIROGRONIANU PRZEZ α-OKSOPOCHODNE LEUCYNY I WALINY W RÓŻNYCH NARZĄDACH SZCZURA

Streszczenie

1. α-Oksoizokapronian i α-oksoizowalerynian powodowały inhibicję spalania α-oksoglutaranu i pirogronianu w mitochondriach; inhibicja była największa w wątrobie, a mniejsza w nerce, sercu i mózgu.

2. W mitochondriach serca szczura rozbitych przez sonikację, α-oksoizokapronian hamował w sposób niekompetycyjny aktywność dehydrogenazy α-oksoglutaranu.

3. Badane związki nie wywierają wpływu na spalanie izocytrynianu i jabłczanu przez mitochondria wątroby.

4. Na podstawie powyższych faktów przypuszcza się, że obserwowana inhibicja spalania α-oksoglutaranu i pirogronianu przez α-oksokwasy o rozgałęzionym łańcuchu węglowym jest w głównej mierze związana z podobnym typem przemian, a co za tym idzie z możliwością kompetycji o wolny koenzym A, lub inhibującym działaniem acylopochodnych koenzymu A na układy dehydrogenaz α-oksoglutarowej i pirogronowej.

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BARBARA PANFIL and A. M. DANCEWICZ

BIOCHEMICAL CHANGES IN LIVER MITOCHONDRIA OF RATS TREATED WITH 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,4,6-TRIMETHYL-PYRIDINE

Department of Radiobiology and Health Protection, Institute of Nuclear Research, ul. Dorodna 16, *Warszawa* 91, *Poland*

1. In liver mitochondria of rats treated with 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDTP), the activity of δ-aminolevulinate synthetase was enhanced, the activities of catalase (EC 1.11.1.6) and ferrochelatase (EC 4.99.1.1) were decreased, and that of cytochrome *c* oxidase (EC 1.9.3.1) was unchanged. No change in mitochondrial respiration was observed; however, when glutamate (but not succinate) was used as substrate, partial uncoupling of oxidative phosphorylation was found to occur. 2. *In vitro*, DDTP had the same effect on the oxidative phosphorylation but it did not affect the activity of the mitochondrial enzymes studied.

Hepatic porphyria can be induced in animals by several substances (see Schmid, 1963). The porphyria-inducing effect of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine[1] was observed by Solomon & Figge (1959) and studied by DeMatteis & Prior (1962). Both in congenital and experimental hepatic porphyria, similar changes were observed: an increase in the activity of ALA-synthetase and accumulation of porphyrins in liver, and increased excretion of porphyrins in urine. Granick (1965) suggested that in congenital and experimental porphyrias, the primary lesion is the excessive biosynthesis of ALA-synthetase. This would indicate that the porphyria-inducing drugs interfere with the mechanism of genetic control of the synthesis of this enzyme. However, the porphyria-inducing substances differ in their chemical structure; on the other hand, the pathway of porphyrin biosynthesis is closely related to other metabolic reactions, and thus other mechanisms possibly involved in the over-production of porphyrins in porphyria should also be considered.

Several observations led to the hypothesis formulated by Labbe (1967) that the inhibition of terminal oxidation by porphyria-inducing substances enhances the synthesis of succinyl-CoA, ALA and further intermediates of porphyrin synthesis.

[1] Abbreviations used: DDTP, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; ALA, δ-aminolevulinate; ALA-synthetase, δ-aminolevulinate synthetase.

It was the purpose of the present work to gain new data which would help to understand the interrelations between the over-production of porphyrins and other metabolic disturbances, especially those of the respiratory chain, appearing in DDTP-induced porphyria.

METHODS

Handling of animals. Adult male Wistar rats were starved for 24 hr., then DDTP suspended in soybean oil was administered intraperitoneally for two successive days with a 24-hr. interval. A dosis of 400 mg. of DDTP per 1 kg. of body weight was used for each injection. The control animals were injected with soybean oil alone.

Twenty four hours after the second injection the rats were killed by decapitation. The livers were excised, washed with cold saline and homogenized at 4° in 0.25 M-sucrose solution using a glass homogenizer with teflon pestle, at 1000 rev./min. Then the mitochondrial fraction was isolated according to Hogeboom (1955). The washed mitochondria were suspended in 0.25 M-sucrose solution; 1 ml. of the suspension contained mitochondria corresponding to 1 g. of liver wet weight (25 - 35 mg. of mitochondrial protein).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using crystalline bovine serum albumin as standard.

Porphyrins were determined in liver homogenates according to the procedure described by Gutniak & Kowalski (1966).

Respiration rate of mitochondria. Oxygen uptake was followed polarographically with an oxygen electrode constructed according to Kahn (1964) and provided with automatic recording. The reaction system described by Chappel (1963) with α-oxoglutarate as substrate, was used. The measurement started with the addition of 0.1 ml. of mitochondrial suspension (about 3 mg. of protein). The results were calculated per 1 mg. of protein per minute.

Oxidative phosphorylation. The manometric technique of Warburg as described by Umbreit, Burris & Staufrer (1967) was employed. The incubation system (2 ml.) was composed of 15 mM-phosphate buffer, pH 7.4, 10 mM-tris buffer, pH 7.4, 5 mM-$MgCl_2$, 1 mM-ATP, 2 mM-EDTA, 2 mM-KF, 10 mM substrate (succinate or glutamate), 56 mM-glucose, 1 mg. of hexokinase and 0.5 ml. of mitochondrial suspension containing approximately 10 mg. of protein in experiments with glutamate and 5 mg. of protein when succinate was used. Glucose and hexokinase were added after 10 min. of temperature equilibration, and the readings of oxygen consumption were made for the next 20 min.; then 1.0 ml. of 20% trichloroacetic acid was added and inorganic phosphate determined according to Fiske & Subbarow (1925).

Enzyme assays. The activity of ALA-synthetase in mitochondria was determined as described by Miyakoshi & Kikuchi (1963) while for determinations in homogenates the method of Marver, Tschudy, Perlroth & Collins (1966), which eliminates the interfering activity of ALA-dehydratase, was applied. After 1 hr. of incubation at 38°, the ALA formed was determined by the method of Mauzerall & Granick (1956) in the modification of DeMatteis & Prior (1962). The activity was expressed in nmoles of ALA formed per 1 mg. of protein per hour or per 1 g. of liver wet weight.

Ferrochelatase activity was assayed by the method of Neuberger & Tait (1964) which is based on the measurement of difference between the amount of protoporphyrins in the samples incubated with and without $FeSO_4$. The results were calculated in nmoles per 1 mg. of protein per hour.

Catalase activity was measured by the method of Euler and Josephson, adapted by Summer & Dounce (1955) to biological preparations. The activity was expressed as the reaction rate constant extrapolated to zero time (k_0) and calculated per 1 mg. of protein.

Cytochrome *c* oxidase activity was measured spectrophotometrically at room temperature as described by Smith (1955) and was expressed as the first-order velocity constant, k, per 1 mg. of protein. The system contained in 2.4 ml. of 0.1 M-phosphate buffer, pH 7.4, 45 nmoles of cytochrome *c* reduced with an equimolar amount of ascorbate. The reaction was started by the addition of 0.1 ml. of diluted mitochondrial suspension containing 0.015 - 0.02 mg. of protein, and E_{550} was measured every 30 seconds. After 3 min., 0.1 ml. of saturated solution of potassium ferrocyanide was added to oxidize completely cytochrome *c*.

Materials. δ-Aminolevulinic acid was synthesized according to Neuberger & Scott (1954). Protoporphyrin from bovine blood was prepared by the method of Grinstein (1947). 3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDTP) was synthesized as described by DeMatteis & Prior (1962). Cytochrome *c* and tris were purchased from Fluka A. G. (Buchs, Switzerland), hexokinase from Nutr. Biochem. Corp. (Cleveland, Ohio, U.S.A.), glutathione from Schwarz Laboratories (New York, N.Y., U.S.A.), ATP from Boehringer & Soehne (Mannheim, West Germany), acetylacetone from T. Schuchardt (Munich, West Germany). All other chemicals were analytical grade of Polish origin.

RESULTS

The results presented in Table 1 show that the administration of DDTP to rats caused a pronounced increase in liver porphyrins and ALA. At the same time, the activity of ferrochelatase in liver mitochondria was decreased and quite

Table 1

The effect of DDTP administration on the concentration of δ-aminolevulinate (ALA) and porphyrins in rat liver homogenates

Experimental porphyria was induced as described under Methods. The number of animals is given in parentheses. Mean values ±S.D. are given in nmoles/g. of liver wet wt.

Animals	ALA	Uro-porphyrin	Copro-porphyrin	Proto-porphyrin
Control (12)	2.5±0.9	0.084±0.035	0.031±0.023	0.071±0.035
Porphyric (9)	6.4±1.0	0.38±0.13	0.58±0.16	17.6±3.4
Increment factor	2.5	4.5	19	248

appreciable activity of ALA-synthetase was observed (Table 2), while in normal liver mitochondria the activity of the latter enzyme was not detectable. The combined effect of changes in the activity of these two enzymes can account for the accumulation of porphyrins in the liver of the treated rats. Besides, the decreased activity of ferrochelatase might lead to a diminished supply of haem for the synthesis of haemoproteins. This possibility was checked by measuring the activity of two haem-containing enzymes: catalase and cytochrome *c* oxidase (Table 3). The activity of catalase in mitochondria of treated rats was lower than in controls while the activity of cytochrome *c* oxidase was unchanged. This suggested that the incorporation of haem into haemoproteins was probably not impaired by DDTP.

Table 2

The effect of DDTP administration on the activities of ALA-synthetase and ferrochelatase in rat liver mitochondria

The enzymes were assayed as described in Methods. The number of experiments is given in parentheses. Mean values ±S.D. are given. The activity of enzymes is expressed as nmoles of ALA formed or protoporphyrin utilized/mg. of protein/hr.

Animals	ALA--synthetase	Ferro-chelatase
Control (9)	not detected	8.2±1.2
Porphyric(18)	0.137±0.015	3.5±1.6

Table 3

The effect of DDTP on the activities of catalase and cytochrome c *oxidase in rat liver mitochondria*

The enzymes were assayed as described in Methods. The number of experiments is given in parentheses. Mean values ±S.D. are given.

Animals	Catalase (k_0/mg. of protein)	Cytochrome *c* oxidase (k/mg. of protein)
Control (9)	0.169±0.03	12.5±3.1
Porphyric (9)	0.079±0.01	12.2±2.8

To study the effect of DDTP *in vitro*, the drug was applied at a concentration of 0.2 mM. This value was chosen as roughly corresponding to that which would be obtained if the DDTP administered to the animals were evenly distributed in their tissues and were not metabolized. The results presented in Table 4 show that *in vitro* DDTP had no effect on the activity of either ALA-synthetase, ferrochelatase or catalase.

Although the activity of cytochrome *c* oxidase was not affected by DDTP *in vivo*, the effect of DDTP on mitochondrial respiration was examined *in vivo* and *in vitro*. The calculated respiration rates for mitochondria from normal rats, with and without the addition of DDTP, and from porphyric rats were practically the same, ranging from 67 to 75 natoms of oxygen/mg. of protein/min.

Table 4

The effect of DDTP in vitro *on the activity of enzymes in liver homogenate and mitochondria*

The enzymes were assayed as described in Methods. Where indicated, the samples contained 0.2 mM-DDTP added *in substantia* or dissolved in 96% ethanol, the final concentration of ethanol being 1.3%. Mean values from 9 separate determinations ± S.D. are given.

Preparation	Addition	ALA-synthetase (nmoles of ALA formed/g. of liver wet wt./hr.)	Ferrochelatase (nmoles of protoporphyrin utilized/mg. of protein/hr.)	Catalase (k_0/mg. of protein)
Homogenate	None, control	41.9±8.2	—	—
	DDTP	38.3±6.0	—	—
Mitochondria	None, control	—	8.2±1.2	0.169±0.032
	1.3% ethanol	—	8.7±2.3	0.128±0.020
	DDTP in ethanol	—	8.4±2.1	0.112±0.020

Table 5

The effect of DDTP in vivo *and* in vitro *on oxidative phosphorylation in rat liver mitochondria*

The assays were carried out by the manometric technique of Warburg as described in Methods. Mean values from 15 separate determinations ±S.D. are given.

Substrate	Mitochondria from	O_2 uptake (μatoms/mg. of protein)	P_i utilization (μmoles/mg. of protein)	P/O ratio
Succinate	Control rat	1.58±0.16	2.74±0.33	1.73
	Control rat, DDTP added	1.47±0.17	2.44±0.60	1.66
	Porphyric rat	1.45±0.10	2.35±0.41	1.62
Glutamate	Control rat	0.30±0.07	0.84±0.16	2.8
	Control rat, DDTP added	0.30±0.04	0.57±0.13	1.9
	Porphyric rat	0.40±0.12	0.81±0.22	2.0

The question then arose whether oxidative phosphorylation was affected by DDTP. The results obtained (Table 5) indicated that DDTP had no effect either *in vivo* or *in vitro* on the phosphorylation coupled with the oxidation of succinate. On the other hand, when glutamate was used as substrate, DDTP inhibited the phosphorylation to about 2/3 of the control value. This indicates that DDTP affected only the first site of oxidative phosphorylation in the respiratory chain.

DISCUSSION

The hypothesis of Granick (1965) that the increase in ALA-synthetase activity in congenital and experimental hepatic porphyria resulted from the induction of synthesis of this enzyme, was confirmed by Labbe, Kurumada & Onisawa (1965), Tschudy *et al.* (1965), Marver, Collins, Tschudy & Rechcigl (1966), Nakao, Wada, Kitamura, Uono & Urata (1966) and Wada, Yano & Urata (1968). Since ALA-synthetase is considered as the enzyme limiting the biosynthesis of porphyrins (Granick, 1962), its induction may account for the increased production of porphyrins. The induction of ALA-synthetase also provides the explanation for the results observed in our experiments.

The increase in ALA-synthetase activity in rat liver mitochondria was observed only when DDTP was administered *in vivo*. The drug applied *in vitro* had no effect on enzyme activity, similarly as it has been demonstrated by Granick & Urata (1963) with guinea pig liver mitochondria.

Ferrochelatase activity in mitochondria was not affected by DDTP *in vitro*, but *in vivo* an inhibition was observed. This effect can be regarded as specific for DDTP action since in experimental porphyria induced by allylisopropylacetamide, Labbe, Hanava & Lottsfieldt (1961) and Onisawa & Labbe (1963) observed no change in the activity of this enzyme. The mechanism of inhibition of ferrochelatase *in vivo* is not clear.

The decrease in the activity of catalase in liver mitochondria *in vivo* seems to be an effect common to porphyria, whether induced by DDTP or other substances, with the exception of hexachlorobenzene (Ockner & Schmid, 1961). Although this effect has not been observed *in vitro*, it seems rather improbable that the supply of haem was impaired, as cytochrome *c* oxidase, which also is a haemoprotein, was not affected.

Cowger, Labbe & Sewell (1963) and Cowger & Labbe (1967) reported that the respiration of L and HeLa cells grown in the presence of some porphyria-inducing drugs, was inhibited. In our experiments with DDTP, regardless whether it was applied *in vivo* or *in vitro*, no changes in respiration were observed in liver mitochondria. It should be noted that the conditions of the two experiments differed widely, and not the same porphyria-inducing substances were used.

The hypothesis of Labbe (1967) that the impairment of the terminal oxidation plays an important role in the action of porphyria-inducing substances was based on the observations that in liver of porphyric rat the activity of NADH oxidase is

decreased (Cowger, Labbe & Mackler, 1962) and the amount of ATP is lowered (Gajdos, Gajdos-Török, Palma-Carlos & Palma-Carlos, 1967). We have shown that DDTP, although it had no effect on respiration, uncoupled the phosphorylation linked to the oxidation of NADH. This result was obtained irrespective whether the drug was applied *in vivo* or *in vitro*. Thus, it seems possible to assume that the decrease of ATP in porphyria is the result of partial uncoupling of oxidative phosphorylation.

Since the inhibition of oxidative phosphorylation amounted at most to 1/3 of the control value, and since no change in respiration of mitochondria was detected, it may be assumed that the impairment, if any, of the respiratory chain and the production of ATP in tissues of rats with porphyria induced by DDTP is small and not relevant to the mechanism of over-production and accumulation of porphyrins. The nature of the induction of porphyria should be rather looked for in the interference of porphyria-inducing substances with the apparatus of genetic control of porphyrin metabolism processes.

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ZMIANY BIOCHEMICZNE W MITOCHONDRIACH WĄTROBY SZCZURA PO PODANIU 3,5-DWUETOKSYKARBONYLO-1,4-DWUHYDRO-2,4,6-TRÓJMETYLOPIRYDYNY

Streszczenie

1. W mitochondriach wątroby szczurów, którym podawano 3,5-dwuetoksykarbonylo-1,4-dwuhydro-2,4,6-trójmetylopirydynę (DDTP), stwierdzono zwiększenie się aktywności syntetazy δ-aminolewulinowej, zmniejszenie aktywności katalazy i ferrochelatazy oraz brak zmian aktywności oksydazy cytochromu *c*. Oddychanie mitochondriów nie uległo zmianie, obserwowano jedynie częściowe rozkojarzenie fosforylacji oksydacyjnej, kiedy jako substratu użyto glutaminianu. W przypadku bursztynianu rozkojarzenia nie stwierdzano.

2. *In vitro* wpływ DDTP na oksydacyjną fosforylację był taki sam, natomiast aktywność enzymów mitochondrialnych w tych warunkach nie była zmieniona.

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RECENZJE KSIĄŻEK

HORMONE AND METABOLIC RESEARCH. Dwumiesięcznik, ok. 64 stron w zeszycie. G. Thieme Verlag, Stuttgart 1969; prenumerata roczna $ 24.—

Do czasopism biochemicznych doszła w roku 1969 nowa pozycja, a mianowicie *Hormone and Metabolic Research*. Naczelnymi redaktorami czasopisma są R. Levine z Nowego Jorku i E. F. Pfeiffer z Ulm, a współredaktorami: S. Berson (Nowy Jork), D. H. Copp (Vancouver), R. I. Dorfman (Stanford), V. F. Foglia (Buenos Aires), R. Fraser (Londyn), W. Gepts (Bruksela), H. Holzer (Fryburg), R. Luft (Sztokholm), L. Martini (Mediolan), J. E. Rall (Waszyngton), A. E. Renold (Genewa), F. Stutinsky (Strasburg), E. Tonutti (Ulm), N. A. Yudaev (Moskwa), H. Zahn (Akwizgran). Nazwiska te są przeważnie dobrze znane biochemikom i endokrynologom, i zapewniają odpowiedni poziom przyjętych prac eksperymentalnych. Artykuły drukowane są w językach: angielskim, francuskim lub niemieckim.

Zamierzeniem redaktorów jest jak najszybsze drukowanie przyjętych prac. Ponadto przyjmowane są również "Short-communications" i "Stop-press-communications", których ukazanie się w druku będzie przyśpieszone przez zastosowanie techniki offsetowej.

W zeszycie pierwszym, ze stycznia 1969 r., umieszczone są następujące prace:

1. HGH and ACTH secretory responses to stress (R. S. Yalow, N. Varsano-Aharon, E. Echemendia i S. A. Berson, Nowy Jork).

2. Secretion of and sensitivity to insulin in obese rats fed a high-fat diet (W. J. Malaisse, D. Lemonnier, F. Malaisse-Lagae i I. M. Mandelbaum, Bruksela i Paryż).

3. Cholinesterase-Aktivität bei der Frühentwicklung der Inselanlagen des Hühnerembryos (U. Drews, E. Kussäther i K. H. Usadel, Ulm).

4. Toxic adenoma of the thyroid (B. Malamos, D. A. Koutras, D. Fringeli i C. N. Tassopoulos, Ateny).

5. The acute insulin synergistic activity of growth hormone. I. Inhibition by chronic growth hormone administration (R. J. Mahler i O. Szabo, Nowy Jork).

6. Thin layer chromatography of urinary glycosaminoglycans as screening procedure for mucopolysaccharidoses (W. M. Teller i A. Ziemann, Marburg).

Short-communications:

1. A modification of the method of determination of the glucose assimilation coefficient (K) (H. F. Schaefer i K. Mendner, Frankfurt nad Menem).

2. The secretion of calcitonin by the perfused ultimobranchial gland of the hen (R. Ziegler, M. Telib i E. F. Pfeiffer, Ulm).

3. Insulinemies maternelles et foetales chez le rat (J. M. Felix, R. Jacquot i B. Ch. J. Sutter, Reims i Strasburg).

Stop-press-communications:

1. Testosterone metabolism in the isolated perfused human foetal liver (K. Demisch, U. Ammedick i W. Staib, Düsseldorf).

2. Enhancement of reactive insulin secretion *in vitro* by cobalt-chloride ($CoCl_2$) (M. Telib i E. F. Pfeiffer, Ulm).

Maszynopisy prac przedstawianych do druku należy przesyłać na adres: Prof. Dr. E. Levine, Dept. of Medicine, New York Medical College, 5th Avenue at 106th Street, New York 10029, N.Y., U.S.A.; lub: Prof. dr. E. F. Pfeiffer, Zentrum für Innere Medizin, Universität Ulm, D 7900 Ulm/Donau, Steinhövelstrasse 9, West Germany.

Irena Mochnacka

SOIL BIOLOGY AND BIOCHEMISTRY. Kwartalnik, ok. 100 stron w zeszycie. Pergamon Press, Oxford 1969; prenumerata roczna £ 12.

Soil Biology and Biochemistry jest nowym czasopismem biochemicznym, którego problematyka obejmuje zagadnienia biologii, ekologii i biochemii wszystkich organizmów glebowych i ich wpływu na wzrost roślin.

W związku z coraz powszechniejszym stosowaniem przez rolnictwo środków chwastobójczych i owadobójczych, które, jak wiadomo, są toksyczne dla żywych organizmów, zaistniała pilna potrzeba połączenia badań nad przemianą tych preparatów w roślinach i glebie oraz nad ich wpływem na skład i metabolizm organizmów glebowych. Potrzebę wydawania czasopisma dotyczącego tych zagadnień wydawnictwo uzasadnia również coraz większą liczbą badaczy zajmujących się biologią i biochemią gleby, których prace były dotychczas publikowane w różnych czasopismach.

Pismo ogłasza oryginalne prace i krótkie doniesienia — zasadniczo w języku angielskim; zamieszczane są również prace w językach francuskim i niemieckim (ze streszczeniami po angielsku).

Problematyka pierwszego zeszytu *Soil Biology and Biochemistry* obejmuje zagadnienia od wpływu herbicydów na rozwój organizmów glebowych, poprzez badania nad aktywnością enzymów glebowych rozkładających polisacharydy i ich pochodne oraz mocznik, do zastosowania promieniowania jonizującego w badaniach nad biologią i biochemią gleby.

Maszynopisy przedstawianych do druku prac należy przesyłać na adres: Professor E. W. Russell, C. M. G., Soil Biology and Biochemistry, Department of Soil Science, The University, Reading, RG1 5AQ, England.

Kazimierz Kleczkowski

Dennis V. Parke, THE BIOCHEMISTRY OF FOREIGN COMPOUNDS. International Series of Monographs in Pure and Applied Biology (Biochemistry Division, Volume 5), Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1968; str. 269; cena 75 s.

Książka stanowi kolejny tom z serii poświęconej wybranym zagadnieniom biochemii. Omawia ona aspekty biochemiczne wybranych związków obcych dodawanych do żywności, leków, pestycydów i niektórych związków stosowanych w przemyśle chemicznym. Monografia ta może być uważana jedynie jako wprowadzenie do toksykologii omawianych grup związków. Posiada ona jednak szereg walorów, oryginalność ujęcia tematu polegającą na całościowym omówieniu zagadnienia związków obcych, dobre przedstawienie elementów biochemii tych związków oraz bardzo dobre ujęcie tematu z punktu widzenia dydaktycznego. Walory te nadają omawianej monografii wysoką rangę.

Monografia składa się z dwu części. W pierwszej, ogólnej, autor omawia mechanizmy biochemiczne absorbcji i wydzielania z organizmu związków obcych oraz ich metabolizm zarówno u człowieka i ssaków, jak i u zwierząt zmiennocieplnych. W pięciu rozdziałach drugiej części omawiane są poszczególne związki i grupy związków — związki pochodzenia naturalnego, syntetyczne substancje dodawane do żywności, niektóre leki, pestycydy oraz niektóre związki stosowane w przemyśle chemicznym, będące przedmiotem zainteresowania higieny pracy.

Nowoczesność omawianej monografii polega na wprowadzeniu do literatury naukowej i syntetycznym omówieniu problemu biochemii związków obcych jako zagadnienia, które wobec coraz

większego schemizowania środowiska życia człowieka i innych organizmów staje się przedmiotem współczesnej biochemii.

Książka jest napisana w sposób przystępny dla niespecjalistów. Przeznaczona jest głównie dla pracowników naukowych i studentów wydziałów rolniczych, medycznych i biochemicznych. Zawiera ona w niewielkiej stosunkowo objętości duży materiał treści, bogato ilustrowany schematami przebiegu reakcji i rycinami. Wykaz cytowanej literatury naukowej wraz z tytułami obejmuje ponad 500 pozycji do roku 1966 włącznie. Książka zawiera indeks rzeczowy i indeks autorów.

Tadeusz Chojnacki

G. Gehrmann, HÄMOLYSE UND HÄMOLYTISCHE ANÄMIEN. G. Thieme Verlag, Stuttgart 1969, str. 323, cena DM 74,—.

Książka Gehrmanna jest monografią o chorobach, w których wiodącym objawem jest hemoliza. Zmianie uległo kryterium hemolizy, pomimo utrzymania się tradycyjnej nazwy. Jako kryterium hemolizy uważa się obecnie skrócenie czasu przeżycia krwinki czerwonej a nie, jak poprzednio, zmniejszenie oporności osmotycznej, przy czym nie ma prostej zależności między tymi dwoma zjawiskami. Choroby hemolityczne stanowią grupę chorób, w których dzięki biochemii dokonano w ostatnich latach ogromnego postępu w zakresie diagnostyki i znajomości patogenezy. Książka prof. Gehrmanna omawia te choroby, uwzględniając zarówno klinikę, jak i serologię, hematologię oraz — w szerokim zakresie — biochemię.

Układ monografii jest zwięzły i logiczny. Składa się ona z dwóch głównych działów: fizjologia i patofizjologia erytrocytów, obejmująca rozdziały: biochemia i kinetyka erytrocytów, chemia, struktura i funkcja błony erytrocytów, starzenie się i destrukcja erytrocytów, ogólne objawy hemolizy; oraz części właściwej — anemie hemolityczne, obejmującej trzy rozdziały: hemolityczne anemie uwarunkowane przyczynami wewnątrzkrwinkowymi, zewnątrzkrwinkowymi oraz anemie spowodowane przyczynami mieszanymi wewnątrz- i zewnątrzkrwinkowymi.

Piśmiennictwo obejmuje około 1100 pozycji do 1968 roku.

Podział na intra- i ekstraerytrocytarnie uwarunkowane anemie jest niewątpliwie nowością i postępem w stosunku do podziałów poprzednich. Intraerytrocytarnie uwarunkowane anemie są dziedziczne, ekstraerytrocytarnie uwarunkowane są nabyte z wyjątkiem nocnej napadowej hemoglobinurii i anemii lekowrażliwych, w których defekt jest wewnątrzkrwinkowy, ale wywołująca przyczyna jest zewnątrzkrwinkowa.

Biochemika nie zajmującego się biochemią kliniczną uderza mnogość stanów hemolitycznych i zmian struktury krwinki czerwonej o przyczynie ściśle biochemicznej, polegającej albo na niedoborach lub brakach aktywności niektórych enzymów, albo na nieprawidłowościach strukturalnych hemoglobiny lub nieprawidłowościach strukturalnych ścianki krwinki czerwonej.

Jedynie dziedziczna sferocytoza (dawny *icterus hemolyticus*), atypowa (dziedziczna) sferocytoza i dziedziczna eliptocytoza mają jeszcze niezbyt jasno zdefiniowaną biochemiczną patogenezę, natomiast grupa dziedzicznych niesferocytowych anemii jest pięknym przykładem postępów biochemii w zakresie kliniki.

Przyczyną hemolitycznej anemii niesferocytowej mogą być następujące defekty enzymatyczne: z cyklu Embdena Mayerhofa dotyczące heksokinazy, izomerazy triozofosforanowej, 2-3 fosfogliceromutazy i kinazy pirogronianowej; z cyklu pentozowego — brak dehydrogenazy glukozo-6-fosforanowej, reduktazy glutationowej, syntetazy glutationowej, dehydrogenazy 6-fosfoglukonianowej oraz — stojący poza tą systematyką — brak ATPazy.

Drugą grupą, w której specyficzny defekt biochemiczny jest przyczyną anemii hemolitycznej, jest grupa talassemi. Jest to genetycznie uwarunkowane upośledzenie syntezy prawidłowej hemoglobiny dorosłych A_1, przy utrzymywaniu się zawartości we krwi hemoglobiny płodowej Hb F albo ze zwiększeniem zawartości hemoglobiny A_2, niekiedy Hb H, Hb Barts lub Hb Lepore. O ile talassemia stanowi przykład zaburzenia syntezy normalnej hemoglobiny, o tyle hemoglobinopatie są przykładami występowania genetycznie uwarunkowanej nieprawidłowej hemoglobiny. Znanych

jest dotychczas 40 nieprawidłowych hemoglobin. z których autor wymienia tylko kilka o bardziej praktycznym klinicznym znaczeniu — hemoglobinopatię S, będącą przyczyną anemii sierpowatej, hemoglobinopatię C z połową zawartości Hb S, różniącej się zawartością Hb S; hemoglobinopatie C, D i E będące wariantami Hb A. W wariantach tych zachodzi podstawienie normalnego aminokwasu w łańcuchu beta kwasem glutaminowym w różnych pozycjach. Dziedziczna anemia z ciałkami Heinza okazała się być hemoglobinopatią Köln. Hemoglobina Köln wykazuje dziwną wrażliwość na ogrzewanie, część jej wytrąca się przy ogrzewaniu w 50°. Podobną niestabilność wykazuje Hb H i Hb Zurich.

Grupa ekstraerytrocytarnych anemii hemolitycznych dotychczas mniej interesuje biochemika niż serologa. Skrócenie okresu przeżycia krwinki czerwonej oparte jest na mechanizmie hemolizy serologicznej — przez izoprzeciwciała A i B, przez przeciwciała nabyte anty Rh, anty Kell itd., przez autoprzeciwciała, jak np. przy napadowej hemoglobinurii z chłodu itd. Duża grupa hemoliz objawowych powstaje w wyniku działania różnych czynników, jak np. fizykalnych, infekcyjnych, termicznych, chemicznych, toksycznych lub opartych na mechanizmie immunologicznym, ew. na mechanizmie niedoboru enzymów, np. niedobór glukozo-6-fosfatazy z jego wariantami genetycznymi czy hemoglobinopatycznymi — Hb H i Zurich.

Książka jest raczej typu klinicznego. Nie daje żadnych szczegółów wykonawczych, metodycznych. Nie mniej jednak jest zbiornicą dobrze uporządkowanych i bardzo aktualnych informacji z odnośnikami do aktualnego piśmiennictwa na dany temat.

Niewątpliwie winna ona znaleźć się w rękach hematologów, internistów, biochemików klinicznych, pediatrów oraz biochemików ogólnych, cytologów i cytochemików.

Leszek Tomaszewski

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS (R. T. Holman, ed.) vol. 10, part 2. G. R. Waller, Metabolism of Plant Terpenoids. Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1969; str. 151—238, cena 37/—, $ 5.00.

Kolejny tom wydawnictwa poświęcony metabolizmowi terpenoidów roślinnych jest napisany przez jednego autora. Omówienie metabolizmu powszechnie znanych związków terpenoidowych musiało być z konieczności ograniczone do tych grup, dla których badania takie były prowadzone głównie metodą podawania prekursorów znakowanych i badania lokalizacji atomów znakowanych w wytworzonych związkach metodami chemicznymi. W mniej licznych przypadkach opisano również procesy enzymatyczne. Stąd pewne dysproporcje w rozdziale materiału.

Po krótkim wstępie, mówiącym o różnorodności terpenoidów i powszechności ich występowania w roślinach, autor w najobszerniejszym ze wszystkich rozdziale II przedstawia biosyntezę jednostki izopentenylowej i mechanizm jej polimeryzacji, a następnie podaje przegląd badań nad biosyntezą monoterpenów, seskwiterpenów, dwuterpenów, trójterpenów i steroli, karotenoidów, alkoholi poliprenoidowych, chinonów poliprenoidowych (tj. ubichinonu, plastochinonu, witamin K i tokoferoli) i wyższych polimerów typu kauczuku i gutaperki. Stosunkowo mało miejsca poświęcono trójterpenoidom, których biosynteza i metabolizm zostały dotychczas najlepiej poznane, gdyż związki te, a zwłaszcza sterydy, były ostatnio przedmiotem szeregu obszernych opracowań monograficznych. Najwięcej miejsca poświęca autor omówieniu syntezy i przemian monoterpenów, a zwłaszcza odkrytych niedawno monoterpenów metylocyklopentanowych zawierających w pierścieniach tlen lub azot. Związki te są obszernie badane w laboratorium autora i badania te doprowadziły między innymi do wykrycia nowej drogi biosyntezy pierścienia pirydynowego w należącej do badanej grupy aktynidynie. W rozdziale III omówiono zależności metaboliczne pomiędzy monoterpenami typu metylocyklopentanowego a pewnymi alkaloidami indolowymi (ajmalicyna, windolina, chinina). W rozdziale IV autor zajmuje się problemem regulacji metabolizmu terpenoidów w roślinach, opierając się głównie na hipotezie Goodwina sugerującej niezależność dróg biosyntezy terpenoidów tak zwanych chloroplastowych i pozachloroplastowych w zależności od oświetlenia.

W rozdziale V przedstawiono katabolizm terpenoidów u zwierząt, bakterii i roślin. W krótkiej konkluzji autor wskazuje na powiązanie pomiędzy metodami chemicznymi, biochemicznymi i fizjologicznymi w badaniu terpenoidów.

Praca Wallera nie opisuje szczegółowo poruszanych problemów ale stara się znaleźć ogólne powiązanie metabolizmu trójterpenów z innymi związkami roślinnymi, zwłaszcza alkaloidami; z tego względu jest cenna nie tylko dla badających terpenoidy, ale i dla wszystkich zajmujących się metabolizmem roślin.

Zofia Kasprzyk

T. Dévényi und J. Gergely, ANALYTISCHE METHODEN ZUR UNTERSUCHUNG VON AMINOSÄUREN, PEPTIDEN UND PROTEINEN. Akadémiai Kiadó, Budapest 1968; str. 347.

Założeniem autorów było podanie takich metod analitycznych badania aminokwasów, peptydów i białek, które można zastosować w każdej pracowni posiadającej podstawowy sprzęt laboratoryjny.

Obecne wydanie jest tłumaczeniem na język niemiecki oryginału węgierskiego wydanego w roku 1963. Autorzy uzupełnili tłumaczenie kilkoma metodami ogłoszonymi w latach 1963-1965.

Książka składa się z 10 rozdziałów dotyczących następujących zagadnień: I. Zakres metod stosowanych przy badaniu białek. II. Badania białek za pomocą elektroforezy niskonapięciowej. III. Wysokonapięciowa elektroforeza bibułowa. IV. Badania immunochemiczne. Otrzymywanie surowicy odpornościowej. V. Metody chromatograficzne. VI. Hydroliza białek i peptydów. VII. Analiza aminokwasów końcowych białek i peptydów. VIII. Oznaczanie białka. IX. Zagęszczanie roztworów białka. X. Chromatografia gazowa pochodnych aminokwasów. Ten ostatni artykuł opracowali R. Kaiser i A. Prox.

Każdy rozdział posiada wstęp teoretyczny i jest zakończony wykazem literatury. Korzystanie z książki przy pracy laboratoryjnej bardzo ułatwi podany przy każdej metodzie zestaw potrzebnej aparatury, szkła i chemikalii oraz dokładny opis postępowania.

Książka będzie bardzo przydatna dla wszystkich pracujących nad białkami i aminokwasami, zarówno w laboratoriach medycznych, jak i chemicznych i biochemicznych.

Irena Mochnacka

NON-INSULIN-PRODUCING TUMORS OF THE PANCREAS. MODERN ASPECTS ON ZOLLINGER SYNDROME AND GASTRIN (L. Demling & R. Ottenjann, eds.) Georg Thieme Verlag, Stuttgart 1969; str. 213, cena 29.- DM.

Wydany pod redakcją prof. Demlinga i doc. Ottenjanna zbiór prac przedstawionych w dniach 16 i 17 lipca 1968 r. na Uniwersytecie w Erlangen na sympozjum dotyczącym guzów trzustki nie wytwarzających insuliny, stanowi interesującą pozycję bibliograficzną. Publikacja zawiera 19 oryginalnych prac badawczych o charakterze zarówno doświadczalnym, jak i klinicznym, przedstawionych przez 32 autorów reprezentujących znane ośrodki gastro-enterologiczne z 7 krajów świata. Każdy referat jest bogato ilustrowany i zaopatrzony w obszerne piśmiennictwo oraz głosy dyskusyjne, które niewątpliwie znacznie rozszerzają omawiane zagadnienie.

Problem guzów trzustki nie wytwarzających insuliny, zwłaszcza problem zespołu Zollingera-Ellisona, stanowi od niedawna szczególny przedmiot zainteresowania gastro-enterologów. Ustalone dotychczas fakty przyczyniły się do wyjaśnienia patogenezy tego stosunkowo rzadkiego schorzenia, a przede wszystkim do poznania fizjologicznych i farmakologicznych własności gastryny, którą przed kilku laty udało się zsyntetyzować.

Ważnym aspektem przewijającym się w wygłaszanych pracach i w dyskusjach nad nimi były próby ogólnego określenia roli gastryny w patogenezie choroby wrzodowej.

Publikacja ma niewątpliwie dużą wartość zarówno dla biochemików, fizjologów, jak i dla klinicystów, interesujących się zagadnieniami gastro-enterologii. Godnym podkreślenia jest fakt, że oceniany zbiór prac wyszedł z druku w niespełna rok od daty sympozjum.

Zbigniew Kaleta

J. Enselme, UNSATURATED FATTY ACIDS IN ATHEROSCLEROSIS (II ed.). Pergamon Press, Oxford 1969; str. 163, tabel 61; cena 90/-, $ 12.00.

Patogeneza miażdżycy nie jest dotąd ostatecznie wyjaśniona mimo ogromnej ilości prac doświadczalnych, klinicznych i epidemiologicznych prowadzonych na całym świecie. W świetle współczesnych badań, etiopatogeneza miażdżycy jest bardzo złożona; istnieje bowiem wiele czynników wrodzonych, nabytych i środowiskowych, które w różnym stopniu wpływają na rozwój miażdżycy. Jednym z czynników przyśpieszających rozwój miażdżycy jest nadmiar tłuszczowców krwi, a zwłaszcza podwyższony poziom cholesterolu, trójglicerydów, nasyconych kwasów tłuszczowych; natomiast nienasycone kwasy tłuszczowe mają hamować rozwój miażdżycy.

Autor omawianej monografii przedstawił całokształt zagadnień dotyczących zaburzeń gospodarki lipidowej, ze szczególnym uwzględnieniem nienasyconych kwasów tłuszczowych, w patogenezie miażdżycy. Pracę swą oparł na ogromnym piśmiennictwie, prawie wyłącznie anglosaskim. W kolejnych rozdziałach omówił on definicję miażdżycy, udział w jej rozwoju cholesterolu i kwasów tłuszczowych. Najwięcej uwagi poświęcił autor budowie i pochodzeniu nienasyconych i nasyconych kwasów tłuszczowych; następnie przedstawił wpływ tłuszczów roślinnych i zwierzęcych na rozwój miażdżycy. Do tłuszczów hamujących rozwój miażdżycy należą np. olej kukurydzy, soji, słonecznika, siemienia lnianego, rzepaku, a także olej ryb i foki; zawierają one bowiem dużo nienasyconych kwasów tłuszczowych. Tłuszcze zwierzęce, zawarte w mleku, maśle, śmietanie, serach, jajach, mięsie, składają się z nasyconych kwasów tłuszczowych i przyśpieszają rozwój miażdżycy.

W dalszych rozdziałach omówiono udział fosfolipidów, mukopolisacharydów i białek w patogenezie miażdżycy.

Oddzielnie przedstawił autor wyniki badań epidemiologicznych przeprowadzonych wśród wielu ras i narodów świata, u których rozpatrywano wpływ odżywiania, ze szczególnym uwzględnieniem rodzai tłuszczów, na częstość występowania miażdżycy. Nie pominięto również wpływu na rozwój miażdżycy niektórych hormonów (płciowych, tarczycy, oraz adrenaliny). Końcowy rozdział poświęcony jest roli prawidłowego odżywiania w zapobieganiu rozwojowi miażdżycy.

Jak wynika z powyższego, autor — wbrew tytułowi pracy — przedstawił nie tylko rolę nienasyconych kwasów tłuszczowych, lecz także pozostałych frakcji tłuszczowców oraz białka w patogenezie miażdżycy. Uwzględnił on również rolę innych czynników, które, wywierając wpływ na skład tłuszczowców krwi, wpływają na rozwój miażdżycy.

Książka jest napisana bardzo jasno i czyta się ją łatwo i z przyjemnością. Zaznajomienie się z tą monografią przyniesie korzyść wszystkim, którzy interesują się zagadnieniem patogenezy miażdżycy, a zwłaszcza rolą tłuszczów. Wśród ogromnego piśmiennictwa zestawionego w poszczególnych rozdziałach nie znalazłem jednak prac polskich autorów, ogłoszonych w czasopismach anglosaskich.

Mieczysław Kędra

LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY (T. S. Work and E. Work, eds.) tom I. North-Holland Publ. Comp., Amsterdam 1969, str. 580.

Tom I monografii pod redakcją małżeństwa Work, który ostatnio pojawił się na półkach księgarskich, obejmuje trzy części poświęcone omówieniu następujących technik laboratoryjnych: elektroforezie białek w żelu skrobiowym i poliakryloamidowym, chromatografii na kolumnach

wypełnionych żelami oraz technikom immunochemicznym stosowanym do identyfikacji i charakterystyki związków makrocząsteczkowych.

Pierwsza część I tomu, jak już wspomniano, jest poświęcona zastosowaniu technik elektroforetycznych w żelach. Opracowana jest przez znanego specjalistę, dr A. H. Gordona, autora licznych prac metodycznych w zakresie chromatografii i elektroforezy. Obejmuje ona pięć rozdziałów (145 stron), w których autor przedstawia wprowadzenie teoretyczne, opisy aparatury, liczne przepisy metodyczne i różne przykłady zastosowania żelów skrobiowych i poliakryloamidowych do analizy i preparatywnego wydzielania białek. Znajduje się tu szereg nowych i użytecznych w praktyce laboratoryjnej przepisów dotyczących takich zagadnień, jak oznaczanie radioaktywności w żelach z opisem urządzeń do cięcia sztabek żelu na skrawki, ich solubilizacji i przygotowania do oznaczenia radioaktywności za pomocą ciekłych scyntylatorów. Podane są również sposoby eluowania i odzyskiwania rozdzielonych substancji z żelów. Autor podaje przykłady licznych rozdziałów w żelach w obecności dodatkowych składników, zwiększających rozdzielczość nośnika, jak np. mocznik, siarczan dodecylu, fenol i inne. Szczególnie dokładnie i fachowo opisane zostały sposoby preparatywnego frakcjonowania białek w żelach syntetycznych. Użytecznym uzupełnieniem tej części książki jest zestawienie najczęściej stosowanych mieszanin buforowych.

W dalszych dwóch częściach podręcznika L. Fischer i J. Clausen omawiają zagadnienia metodyczne związane z chromatografią na kolumnach wypełnionych żelami oraz dotyczące różnych technik immunodyfuzyjnych i immunoelektroforetycznych stosowanych przy badaniu białek.

W podręczniku położono nacisk raczej na szczegółowe przepisy metodyczne, podając liczne przykłady zastosowania tych metod, niż na wprowadzenia teoretyczne. Dlatego podręcznik państwa Work znajdzie szerokie zastosowanie jako użyteczny przewodnik w każdym laboratorium zajmującym się analizą i badaniami własności substancji białkowych, bez konieczności odnoszenia się do oryginalnych prac rozsianych po licznych czasopismach specjalistycznych. Każda część książki posiada zebrane piśmiennictwo do r. 1967 oraz szczegółowy indeks rzeczowy. Liczne rysunki, zdjęcia i tabele nadają przyjemną graficzną szatę temu cennemu wydawnictwu.

Włodzimierz Ostrowski