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THE ROLE OF LIPIDS IN FERROCHELATASE ACTIVITY

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1. Ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1) activity in rat liver mitochondria extracts of low specific activity is stimulated by crude lipid extracts, neutral lipids, phosphatidylethanolamine and acidic phospholipids. 2. The inhibitory effect of albumin on ferrochelatase could be partially overcome by lipids or long chain fatty acids. 3. Peroxide-containing lipids lost their ability to stimulate the activity of ferrochelatase preparations of low specific activity. Oxidation of mitochondrial lipids to peroxides led to inactivation of the enzyme.

The involvement of lipids in catalytic activity of ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1) has been suggested by Yoshikawa & Yoneyama (1964) and by Mazanowska, Neuberger & Tait (1966). Some observations made in our Laboratory during studies on the isolation and purification of the enzyme were also in agreement with this suggestion (Mazanowska, Dancewicz, Malinowska & Kowalski, 1969). The present communication demonstrates that lipids are required for ferrochelatase activity and that changes in the lipid component of the enzyme may affect its activity.

MATERIALS AND METHODS

Enzyme preparations were suspensions of mitochondria in 0.1 M-tris-HCl buffer, pH 8.4, or Tween 20 extracts of rat or guinea-pig mitochondria prepared as described previously (Mazanowska *et al.*, 1969).

Crude lipid preparations were obtained from chromatophores of *Rhodopseudomonas spheroides* or from rat liver mitochondria. Chromatophores were prepared according to Gibson, Neuberger & Tait (1963) from the organisms grown semi-anaerobically in the light in the malate medium of Lascelles (1959). Chromatophores were treated with Tween 20 added to the final concentration of 1%, left frozen overnight, then thawed and centrifuged at 105 000 *g* for 1 hr. The supernatant was

discarded, and the dark-brown pellet suspended in 0.05 M-tris-HCl buffer, pH 8.4, was used as the lipid preparation. Liver mitochondria were extracted with a chloroform-methanol mixture according to Bligh & Dyer (1959). The extract was evaporated to dryness under a stream of nitrogen and the residue dissolved in a small volume of chloroform (1/100 of the original extract volume).

Column chromatography of mitochondrial lipids was performed on silicic acid activated before use by heating overnight at 100°. A sample of concentrated lipid solution in chloroform, equivalent to mitochondria obtained from 1 - 2 g. of liver, was applied to the column (1 × 14 cm.) of 5 g. of silicic acid, and eluted successively with chloroform and methanol, using three column volumes of each solvent. Both fractions were evaporated to dryness and redissolved in chloroform (2.5 volumes of that applied to the column); 2/3 volume of the fraction eluted with methanol was rechromatographed on another silicic acid column. This time the elution was performed successively with chloroform, then with stepwise increasing concentrations of methanol (4, 10, 20, 60 and 80%) in chloroform, and finally with methanol; the amount of solvent at each step corresponded to two and a half column volumes. Each fraction was evaporated to dryness and dissolved in chloroform (2.5 volumes of that applied to the column).

Chloroform solutions of all fractions were analysed for lipid composition, phosphate content and ability to stimulate ferrochelatase activity.

Thin-layer chromatography of lipids was performed on 5 × 8.5 cm. glass plates coated with Kieselgel G and developed with a mixture of chloroform - methanol - water (65:25:4, by vol.) according to Wagner, Hörhammer & Wolff (1961) in the case of phospholipids or with light petroleum (b.p. 45 - 66°) - ethyl ether - acetic acid (60:40:2, by vol.) according to Mangold & Malins (1960) in the case of neutral lipids. The spots were located by spraying with 10% phosphomolybdic acid in ethanol followed by heating at 80 - 82° for 15 min. (Stahl, 1962). The lipids were identified by comparison of their R_F values with those of standards or with R_F given in the literature (Lepage, 1964).

Phospholipid phosphate was determined according to Baginski, Foà & Zak (1967).

Lipid peroxide formation in lipid extracts from mitochondria. Concentrated lipid solution from rat liver mitochondria, 1 ml., was evaporated to dryness, dissolved in 0.2 ml. of anhydrous ethanol, mixed with 1.8 ml. of 0.01 M-tris-HCl buffer, pH 8.4, supplemented to 0.5 mM with ascorbic acid and incubated in air at 37° for 1 hr. Then the amount of lipid peroxides formed was determined. The control sample was incubated without ascorbic acid.

Lipid peroxide formation in mitochondrial preparations. To 0.5 ml. (equivalent to 8 - 9 mg. of protein) of rat liver mitochondria suspension in 0.1 M-tris-HCl buffer, pH 8.4, or the same amount of mitochondrial Tween 20 extracts, different amounts of ascorbic acid and/or 0.1 µmole of $FeSO_4$ were added and supplemented with tris buffer to the final volume of 2.5 ml. This mixture was then incubated in air at 37° for 1 hr. and the content of lipid peroxides formed was determined.

Lipid peroxide content was estimated according to Ottolenghi (1959), the colour formed with 2-thiobarbituric acid being measured at 535 mμ.

Ferrochelatase activity was assayed according to Neuberger & Tait (1964). Protoporphyrin and iron in equimolar amounts were used as substrates. The consumption of protoporphyrin converted to haem was taken as a measure of enzyme activity.

Protein concentration was determined according to Lowry, Rosebrough, Farr & Randall (1951). Protein in the column effluent was estimated at 280 mμ.

Chemicals. Phosphatidylcholine and phosphatidic acid (from egg yolk) were kindly offered by Dr. Halina Dominas, Nencki Institute of Experimental Biology, Warszawa. Protoporphyrin solution was prepared and its concentration determined as described previously (Mazanowska *et al.*, 1969). Silicic acid (100 mesh) was purchased from Mallinckrodt Chemical Works (St. Louis, Mo., U.S.A.). Kieselgel G was a product of E. Merck A. G. (Darmstadt, West Germany). Tween 20 was from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., England). Bovine plasma albumin was obtained from Armour & Co. Ltd. (Eastbourne, Sussex, England). Human plasma albumin was kindly supplied by the Institute of Haematology (Warszawa). All other chemicals were commercial products of analytical grade.

RESULTS

Effect of lipids on ferrochelatase activity

Our first observation indicating that lipid is indispensable for the activity of ferrochelatase was made during attempted purification of the enzyme from mitochondrial Tween extracts by column chromatography on cellulose ion-exchangers. The recovered activity (Fig. 1) was much lower than that of the starting material. However, addition of a crude lipid preparation from chromatophores of photosynthesizing bacteria *R. spheroides* restored the activity of ferrochelatase. A similar effect was observed after addition of lipid extracts from rat or guinea-pig liver mitochondria. The effect of lipids was the greater, the lower was the specific activity of the enzyme preparation used.

To find the lipid compound responsible for the stimulatory effect, the chloroform solution of rat liver mitochondria lipids was chromatographed on silicic acid column, and the obtained fractions were tested for their effect on ferrochelatase activity and analysed by thin-layer chromatography on Kieselgel G. The obtained data (Table 1) indicate that fractions which contain as a main component either neutral lipids, phosphatidylethanolamine or acidic phospholipids cause a 2 to 2.5-fold increase in specific activity of a weakly active (1.9 nmoles of haem/mg. protein/hr.) ferrochelatase preparation. The fraction containing phosphatidylcholine as a main component was inactive causing in fact some inhibition of the enzyme activity.

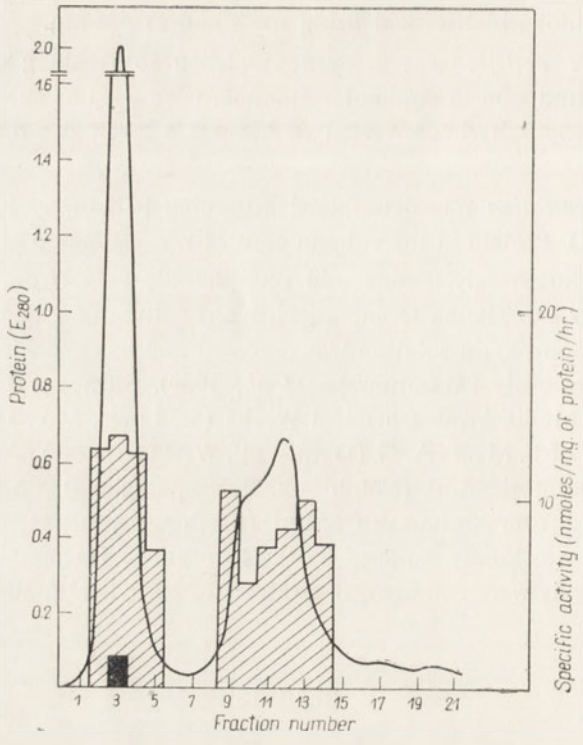

Fig. 1. DEAE-cellulose column chromatography of ferrochelatase preparation. The 0.2 - 0.4 ammonium sulphate saturated fraction of Tween 20 extract from guinea-pig liver mitochondria (57.6 mg. protein) was used as starting material. The column (1.0 × 62.0 cm.) was equilibrated with 0.01 M-Na, K-phosphate buffer, pH 7.4, and the protein was eluted with a linear NaCl concentration gradient up to 0.5 M. Fractions of 2.5 ml. were collected at a flow rate of 25 ml./hr. Black area, specific activity measured directly; hatched area, specific activity after addition of lipid preparation from chromatophores of *Rhodopseudomonas spheroides*. The activity of the starting material measured directly was 10.7, and after addition of the lipid extract 16.3 nmoles of protoporphyrin converted to haem/mg. protein/hr.

It seemed of interest to see whether phospholipids from another source would be able to stimulate ferrochelatase activity. Therefore phosphatidic acid and phosphatidylcholine from egg yolk, and free fatty acids were used. The results presented in Fig. 2 show that the effect of these substances was dependent on their concentration. At lower concentrations, phosphatidic acid and sodium laurate stimulated the enzyme activity, then, with increasing concentrations, their effect was gradually reduced and at still higher concentrations they even acted as inhibitors. Stimulation by oleate increased steadily with its increasing concentration, whereas phosphatidylcholine had but a slight, very gradually decreasing, enhancing effect.

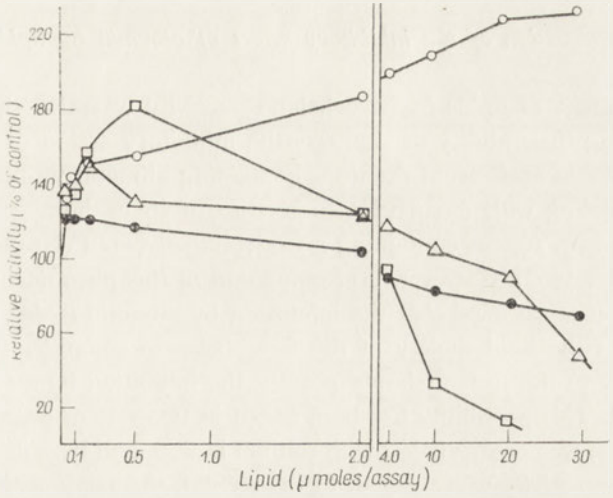

Fig. 2. Effect of lipids on ferrochelatase activity of Tween 20 extracts from rat liver mitochondria. Enzyme activities are expressed in relation to the control (determined without lipids) taken as 100. (○), Sodium oleate; (□), phosphatidic acid isolated from egg yolk; (△), sodium laurate; (●), phosphatidylcholine isolated from egg yolk.

Table 1

Effect of lipids from rat liver mitochondria on ferrochelatase activity

Lipid extract from rat liver mitochondria was prepared and fractionated as described under Materials and Methods. The fractions were analysed, and tested for their effect on ferrochelatase activity. To the assay sample, lipids were added in an amount equivalent to the volume of the mitochondrial suspension, indicated in the Table. The activity of ferrochelatase is expressed in relation to the test without lipid added (control), taken as 100.

Lipid fraction added	Main component	Lipid added equivalent to		Relative activity
		vol. of mitochondrial suspension (ml.)	phosphate (μg. P)	
None (control)				100
Crude extract	all	2.5	10.6	160
Extract from silicic acid column; fractions eluted with:				
chloroform	neutral lipid	0.5	0.05	263
methanol	phospholipids	0.5	15.76	137
Rechromatography of the methanol extract; fractions eluted with:				
4% methanol in chloroform	phosphatidic acid and cardiolipin	0.2	1.15	237
20% methanol in chloroform	phosphatidyl-ethanolamine	0.2	7.4	207
80% methanol in chloroform	phosphatidylcholine	0.2	2.65	78

Suppression of the inhibitory effect of albumin by lipids

The involvement of lipids in ferrochelatase activity was also shown indirectly by demonstrating their ability to suppress the inhibitory action of albumin. This property of albumin and serum fractions containing albumin was reported in our earlier paper dealing with ferrochelatase activity of tissue homogenates or mitochondria tested in a system containing no organic solvents (Mazanowska, Dancewicz & Kowalski, 1962). However, no explanation of this phenomenon was offered at that time. If it is assumed that the inhibition by albumin is due to the binding of fatty acids of the lipid moiety of the ferrochelatase complex, the addition of exogenous lipids to the system should prevent the inhibition by a simple competition mechanism. This possibility has been tested in two sets of experiments. In the first one, albumin was preincubated with sodium laurate and then its ability to inhibit ferrochelatase activity was assayed. In the second, the assay of the enzyme activity was carried out in a system containing sodium laurate and albumin without preincubation. Results presented in Table 2 show that irrespective whether albumin was preincubated with the fatty acid or not, its addition markedly diminished the inhibitory effect of albumin after a certain treshold concentration of fatty acid had been reached. This suppression of the albumin action was proportional to the concentration of the long chain fatty acid.

It should be noted that in these experiments ether was omitted from the incubation mixture because in its presence no inhibition by albumin was observed.

Table 2

The effect of albumin and sodium laurate on ferrochelatase activity of Tween 20 extracts from rat liver mitochondria

Bovine blood serum albumin or human plasma albumin and sodium laurate were added to the ferrochelatase assay system (ether being omitted) and after 1 hr. of incubation at 37° the enzyme activity was determined. In experiments with preincubation, albumin was mixed with sodium laurate and incubated for 1 hr. at 37° prior to the addition to the ferrochelatase assay system.

Components added		Ferrochelatase activity in a system	
Serum albumin (μM)	Sodium laurate (μM)	with preincubation	without preincubation
		nmoles/mg. protein/hr.	
None	None	4.93	
Human, 20	None	0.97	1.09
Human, 20	80	1.23	1.05
Human, 20	160	2.43	2.10
Bovine, 20	None	1.30	1.49
Bovine, 20	80	1.39	1.49
Bovine, 20	160	2.75	2.43

Effect of lipid peroxides on ferrochelatase activity

To obtain different amounts of mitochondrial lipid peroxides, the mitochondrial suspension was incubated in air or in vacuum at 37° for 1 hr. with different concentrations of ascorbic acid, with Fe^{2+} ion at 0.04 mM concentration, or both. The highest oxidation was obtained in air at 0.4 mM concentration of ascorbic acid in the presence of ferrous ions (Table 3). The increase in the amount of peroxides formed diminished ferrochelatase activity leading to a complete or almost complete inactivation of the enzyme.

Table 3

The effect of lipid peroxides formed in mitochondria on ferrochelatase activity

Various amounts of lipid peroxides formed were obtained by incubating mitochondrial suspensions in 0.1 M-tris-HCl buffer, pH 8.4, for 1 hr. at 37° with the indicated compound(s). Ferrochelatase activity and the content of peroxides were determined as described under Materials and Methods.

Incubation conditions for formation of lipid peroxides	Lipid peroxides formed (ΔE_{535}/mg. protein)	Ferrochelatase activity (nmoles/mg. protein/hr.)
Incubated in vacuum		
control (non-oxidized)	0.007	14.1
with 0.04 mM-Fe^{2+}	0.023	13.8
with 0.04 mM-Fe^{2+} + 0.4 mM-ascorbic acid	0.092	6.8
Incubated in air		
with 2 mM-ascorbic acid	0.115	4.1
with 0.4 mM-ascorbic acid	0.163	4.0
with 0.04 mM-Fe^{2+} + 0.4 mM-ascorbic acid	0.359	0.0

Table 4

The effect of lipid peroxides content in lipid extract on its ability to stimulate ferrochelatase activity

Lipid extract from rat liver mitochondria was oxidized by incubation in air as described under Materials and Methods and then its ability to stimulate ferrochelatase activity of Tween 20 mitochondrial extract was compared with that of non-oxidized lipid extract.

Lipid extract added	Lipid peroxides content (ΔE_{535}/mg. protein)	Ferrochelatase activity	
		nmoles/mg.protein/hr.	% of control
None	—	5.1	100
Non-oxidized	0.034	7.0	137.3
Oxidized	0.132	5.8	113.7

Similar results were obtained in experiments in which mitochondrial Tween 20 extracts were used instead of mitochondrial suspension.

The effect of lipid peroxides on the activity of ferrochelatase has been shown also in an experiment in which the ability to stimulate ferrochelatase of low specific activity by extracts of exogenous mitochondrial lipids, oxidized and non-oxidized, were compared (Table 4). The effect of lipid preparations treated with ascorbic acid was much lower than that of the non-treated preparations.

DISCUSSION

Many membrane-bound mitochondrial, microsomal or lysosomal enzymes show an absolute requirement for lipids, both specific and non-specific ones (Green & Fleischer, 1963; Jurtshuk, Sekuzu & Green, 1963; McConnell, Tzagoloff, MacLennan & Green, 1966; Cerletti, Giovenco, Giordano, Giovenco & Strom, 1967; Bulos & Racker, 1968; Duttera, Byrne & Ganoza, 1968). The important role of lipids for mitochondrial ferrochelatase activity has been recognized for some time. Yoshikawa & Yoneyama (1964) were first to note that phospholipase-treated mitochondria lost completely their ferrochelatase activity and that protoporphyrin bound to phospholipids was more readily converted into haem than free protoporphyrin. Mazanowska *et al.* (1966) showed that treatment of mitochondria with acetone or other lipid solvents decreased significantly ferrochelatase activity, which could be restored by the addition of lipid preparation. Mazur (1968) also observed that phospholipid was required for full ferrochelatase activity. In the present study it was shown that the activity of liver mitochondrial ferrochelatase of low specific activity could be stimulated by various lipid compounds. The activating effect was observed with crude lipid extracts from liver mitochondria, chromatophores of *R. spheroides*, as well as with mitochondrial neutral lipids, phosphatidylethanolamine and acidic phospholipids, phosphatidic acid from egg yolk, and free saturated and unsaturated long chain fatty acids, but not with phosphatidylcholine.

Recently similar results were obtained by Sawada, Takeshita, Sugita & Yoneyama (1969) on ferrochelatase preparations of low activity obtained from chicken erythrocyte stroma.

Activation of ferrochelatase by a wide variety of lipid compounds indicates that its requirement for lipid is not very specific. It may be assumed that lipid in ferrochelatase acts in more than one way. It may serve as a reaction medium for substrate and/or it may serve as ligand indispensable for the enzyme protein to preserve its biologically active conformation. These possibilities of lipid involvement in enzyme structure and function were discussed by Green & Fleischer (1963). McConnell *et al.* (1966) using electron microscopy demonstrated that acidic phospholipid was absolutely required by cytochrome *c* oxidase to form vesicle-like structures typical of the active form of the enzyme.

Further evidence for the effect of lipids on ferrochelatase activity is the suppression by fatty acid of the inhibition caused by albumin. The inhibitory action of

albumin may consist in its ability to bind fatty acids of the enzyme. The fact that long chain fatty acids are more readily oxidized when bound to albumin than in the free form (Björntorp, 1966) seems also to be pertinent to the inhibitory action of albumin on ferrochelatase.

The oxidation of mitochondria or mitochondrial Tween 20 extracts by exposure to air during chromatography, diminished their ferrochelatase activity, which could be restored by the addition of lipids. It is thus reasonable to assume that during oxidation of mitochondria the lipid component of the enzyme becomes readily oxidized losing its ability to co-operate with the protein moiety. The observation that peroxide-containing lipids were almost unable to stimulate ferrochelatase activity, also points to such a mechanism being operative.

It may be deduced from the results discussed that the ferrochelatase in its native form is closely associated with lipid, occurring most probably as a lipoprotein bound to the mitochondrial structure. Changes in the lipid component of the enzyme, such as its partial removal by lipid solvent or lipid peroxide formation, result probably in the alteration of the protein moiety of the enzyme followed by a marked decrease in activity.

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ROLA LIPIDÓW W CZYNNOŚCI FERROCHELATAZY

Streszczenie

1. Nieoczyszczone preparaty lipidów, obojętne lipidy, fosfatydyloetanoloamina i kwaśne fosfolipidy stymulują czynność ferrochelatazy (ferro-liazy protohemu, EC 4.99.1.1) w ekstraktach z mitochondriów wątroby szczura posiadających niską aktywność właściwą.

2. Dodanie lipidów lub wyższych kwasów tłuszczowych częściowo znosi hamowanie czynności ferrochelatazy przez albuminę.

3. Lipidy zawierające nadtlenki tracą zdolność stymulacji czynności ferrochelatazowej preparatów o niskiej aktywności właściwej. Utlenianie lipidów mitochondrialnych do nadtlenków powoduje unieczynnienie enzymu.

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THE VARIATIONS OF TRITERPENOIDS IN GERMINATING SEEDS OF *CALENDULA OFFICINALIS*

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The changes in concentration of triterpenic alcohols, oleanolic acid and sterols, free, in the form of esters and glycosides were investigated in germinating seeds of *Calendula officinalis*.

It has been shown by Kasprzyk & Fonberg-Broczek (1967) that the seeds of *Calendula officinalis* contain sterols and oleanolic acid in greater quantities as well as triterpenic monols and diols in smaller but still considerable amounts. Oleanolic acid and sterols are present also in all parts of the calendula plants but of the triterpenic monols only β-amyrin, and of diols only erythrodiol, the metabolic precursors of oleanolic acid, have been identified in the green parts of the plant (Kasprzyk & Wojciechowski, 1969). These two alcohols were found in very small quantities as compared with oleanolic acid. In the flowers of calendula plants, however, four monols and four diols belonging to other than β-amyrin type of pentacyclic triterpenes, are accumulated. In the group of monols, ψ-taraxasterol has been identified by Stevenson (1961) and α-amyrin, taraxasterol and lupeol by Kasprzyk & Pyrek (1968). In the group of diols, arnidiol and faradiol have been identified by Zimmermann (1946) and brein and calenduladiol by Kasprzyk & Pyrek (1968). Sterols present in the flowers of calendula are β-sitosterol, stigmasterol and isofucosterol (Kasprzyk & Turowska, 1969), and they occur in the form of free compounds, acetates and monoglucosides (Kasprzyk, Turowska & Baranowska, 1969). In the group of triterpenes, oleanolic acid was found in calendula plants only in the form of glycosides but triterpenic alcohols are present as free compounds or as acetates. Kasprzyk, Pyrek & Turowska (1968) have found that also sterols occur in the seeds of calendula as free compounds, acetates or glucosides, and that during germination the content of sterols decreases in all fractions.

The aim of the present work was to investigate the types of triterpenic alcohols and sterols present in the seeds of calendula, the forms in which they occur, and changes in their quantities during germination. Changes in the amount of oleanolic acid were also studied.

MATERIAL AND METHODS

Cultivation of plants. The investigated material comprised seeds and 5, 10, 15 and 20-day-old seedlings of *Calendula officinalis* var. Radio. The seeds were germinated on cotton-wool saturated with tap water in a lumistate where they were illuminated 14 hr. per day with the light of 2500 lux intensity. The temperature during the day was 25° and at night 15°. The seeds which did not germinate were removed after 5 days and the seedlings (without shells) placed on paraffin-covered gauze extended over a beaker with the nutrient solution (Kasprzyk & Fonberg-Broczek, 1967) diluted tenfold with water.

Extraction procedure. Seeds and seedlings were counted and weighed. For dry weight determination, samples of 1 g. were used. For extraction, from 50 g. to 250 g. samples were taken, methanol was added so as just to cover the plant material, and homogenized in a Unipan 302 homogenizer at 10 000 rev./min. for 5 min. The homogenate was filtered and the residue heated three times for 10 min. with a fivefold volume of methanol in relation to the weight of the sample; the extracts were combined.

Fractionation of the extract. The methanol extracts were concentrated by distillation to 50 ml. and then 50 ml. of water was added. This was followed by fivefold extraction with 25 ml. portions of light petroleum (b.p. 40 - 60°). By this procedure two fractions were obtained: the light petroleum extract containing free and esterified triterpenoids, and the water-methanol layer containing triterpenoid glycosides.

Separation of free and esterifed triterpenoids. The light petroleum extract was fractionated on column with alumina of III activity (Woelm, Eschwege, W. Germany) using light petroleum with an increasing ethyl ether concentration gradient (up to 100%). The results of separation were checked by thin-layer chromatography.

Hydrolysis of bound triterpenoids. Triterpenoid esters isolated by alumina column chromatography were hydrolysed with 10% KOH solution in methanol for 3 hr. under reflux and the non-saponifying fraction was obtained in the usual way. Triterpenoid glycosides were hydrolysed with 10% H_2SO_4 solution in methanol under the conditions described above.

Thin-layer chromatography. This was performed using silica gel, alumina (both from Merck, Darmstadt, W. Germany) and silica gel impregnated with $AgNO_3$ (10% with respect to gel). The plates were activated at 120° for 30 min. The solvents used were: *I*, light petroleum (b.p. 40 - 60°) - chloroform - methanol (20:10:1, by vol.); *II*, chloroform - methanol (95:5, v/v), for silica gel; *III*, heptane - benzene - ethanol (100:100:1, by vol.), for alumina; *IV*, chloroform; *V*, benzene, for silica gel impregnated with $AgNO_3$. As standards were used: β-sitosterol (Hoffmann, La Roche, Basel, Switzerland), stigmasterol (Sigma, St. Louis, Mo., U.S.A.), as well as β-amyrin, taraxasterol, ψ-taraxasterol, lupeol, faradiol, brein, calenduladiol, oleanolic acid isolated from *C. officinalis* flowers (Kasprzyk & Pyrek, 1968) and erythrodiol (Kasprzyk & Wojciechowski, 1969).

Quantitive determination of triterpenoids. This was carried out using $CoCl_2$ as a complexing agent, according to the method described by Fonberg & Kasprzyk

(1965). Standard curves were made for the mixtures of triterpenic monols and diols (Kasprzyk & Pyrek, 1968) and for the mixture of sterols (Kasprzyk *et al.*, 1969) isolated from flowers of calendula. It proved possible to use the mixture of these compounds as standards because it was demonstrated (see below) that the same sterols and triterpenic alcohols were present in seeds as in flowers.

A comparison of extinction value of $CoCl_2$ complexes of β-sitosterol, ψ-taraxasterol and faradiol with those for the mixtures of sterols, triterpenic monols and triterpenic diols is presented in Table 1. The extinction values for the mixtures were distinctly lower than those for individual compounds tested, the maxima being shifted towards longer wavelengths.

All determinations were made on 3 separate series of seeds and seedlings. The differences between determinations in different series did not exceed 10%.

Table 1

The extinction values of complexes of standard samples of β-sitosterol, ψ-taraxasterol and faradiol, and of mixtures of sterols, triterpenic monols and triterpenic diols isolated from the flowers of Calendula officinalis

The mixture of sterols was obtained as described by Kasprzyk *et al.* (1969), and mixtures of triterpenic monols and diols as described by Kasprzyk & Pyrek (1968).

Compounds (100 µg. in 4.5 ml.)	E_{max} (mµ)	E_{1cm} at E_{max}
β-Sitosterol	260	0.54
Mixture of sterols	265	0.42
ψ-Taraxasterol	250	0.70
Mixture of triterpenic monols	255	0.65
Faradiol	255	0.59
Mixture of triterpenic diols	260	0.53

RESULTS

The extraction method applied previously by Kasprzyk *et al.* (1968) did not allow to achieve the separation of free and esterified sterols, a part of sterols being extracted together with sterol esters. In the present work, efficient separation of free and ester-bound triterpenoids was obtained by chromatography on alumina column. The separated fractions were identified by thin-layer chromatography (Fig. 1). It appeared that triterpenic monols and sterols occur in seeds and seedlings in the form of free and esterified compounds. On the other hand, triterpenic diols were found to occur mostly as esters, free triterpenic diols being present in trace amounts. Similar relations between free and esterfied triterpenoids were observed in the flowers of calendula (Kasprzyk *et al.*, 1969).

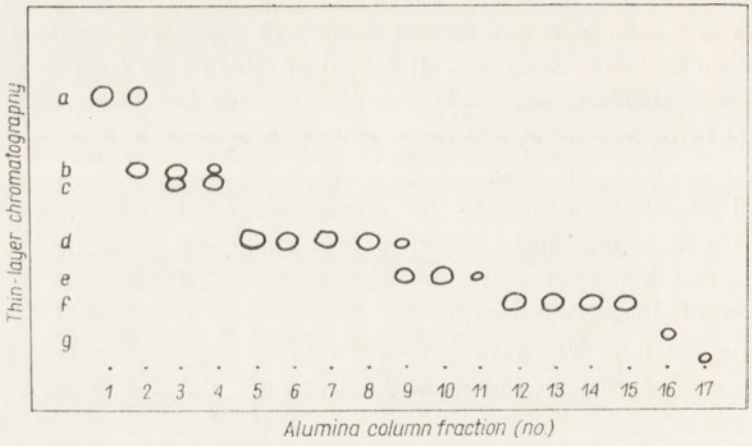

Fig. 1. Thin-layer chromatography on silica gel of free and ester-bound triterpenoids, separated by alumina column chromatography from light-petroleum extracts of *Calendula officinalis* seeds. Solvent system *I*; for details see Methods. The same results were obtained by thin-layer chromatography on alumina with solvent system *III*. *a*, Squalen; *b*, ψ-taraxasterol acetate; *c*, β-sitosterol acetate; *d*, faradiol diacetate; *e*, ψ-taraxasterol; *f*, β-sitosterol; *g*, faradiol.

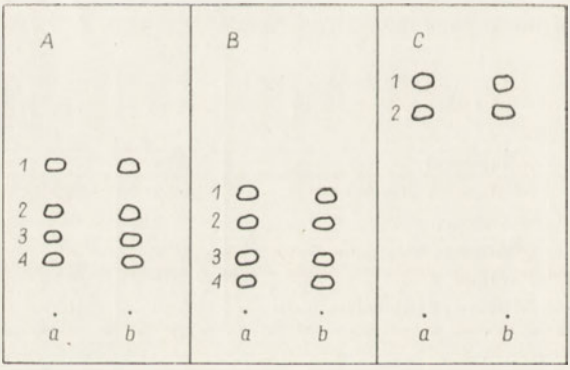

Fig. 2. Separation of *A*, triterpenic monols, *B*, triterpenic diols (diacetates) and *C*, sterols (acetates) from *Calendula officinalis* seeds, by thin-layer chromatography on silica gel impregnated with $AgNO_3$. *A*, Solvent system *IV*: *1*, α- and β-amyrin; *2*, ψ-taraxasterol; *3*, taraxasterol; *4*, lupeol. *B*, Solvent system *V*: *1*, erythrodiol; *2*, brein; *3*, faradiol and arnidiol; *4*, calenduladiol. *C*, Solvent system *IV*: *1*, β-sitosterol; *2*, stigmasterol. *a*, Standards; *b*, sample.

The fraction of triterpenoid glycosides after acid hydrolysis contained sterols and oleanolic acid, as demonstrated by thin-layer chromatography in system *II*.

On the basis of chromatography on silica gel impregnated with $AgNO_3$ and comparison with standards of the individual triterpenoid compounds isolated and identified previously in the flowers of calendula (Kasprzyk & Pyrek, 1968), it was found that all triterpenic monols (Fig. 2A) and triterpenic diols (Fig. 2B) present in flowers occur also in seeds. Moreover, β-sitosterol and stigmasterol (Fig. 2C)

are present in seeds, both compounds occurring as free alcohols, glucosides and esters. Seeds contain also traces of Δ^7 sterols. A similar analysis made on seedlings indicated that all triterpenic monols and diols present in the seed, except β-amyrin and erythrodiol, disappeared completely between the 15th and 20th day of germination. However, all sterols present in seeds were found also in seedlings.

Changes in fresh and dry weight of 1000 seeds or seedlings during germination are shown in Fig. 3. Both the fresh and dry weight of the seedlings increased several-fold in the period between the 5th and 20th day. Dry weight of 1000 seedlings up to the 15th day of germination is lower than that of 1000 seeds. Rapid decrease of dry weight is connected with rejection of the shells and with considerable utilization of storage substances for energy supply. In calendula seedlings, first leaves are formed between the 15th and 20th day of vegetation, and at this time photosynthesis caused

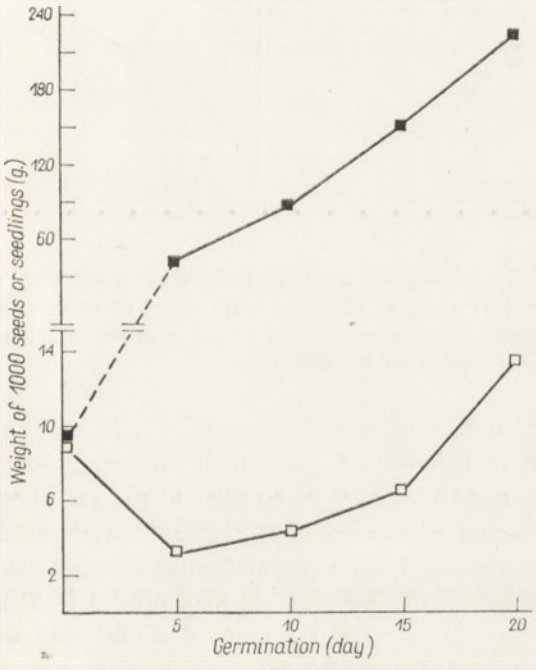

Fig. 3

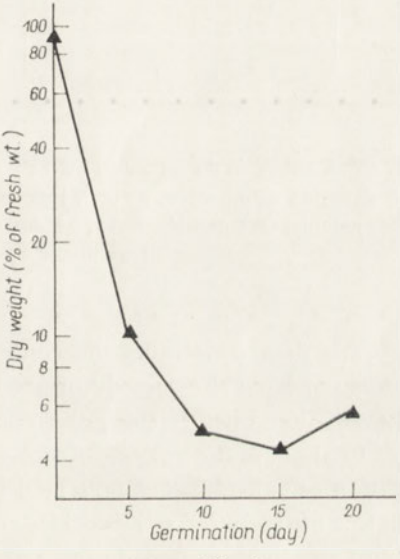

Fig. 4

Fig. 3. Changes in (■), fresh and (□), dry weight of 1000 seeds or 1000 seedlings of *Calendula officinalis* during germination

Fig. 4. Relation of dry to fresh weight of *Calendula officinalis* seeds and seedlings during germination.

a double increase of dry weight of the seedlings. The increase of dry weight observed in the period 5th to 15th day, results from photosynthetic activity of the cotyledons. The dry weight as percentage of fresh weight is shown in Fig. 4. The relative increase of dry weight occurring between the 15th and 20th day indicates that the rate of biosynthesis of organic substances in young leaves during this period was greater than their consumption.

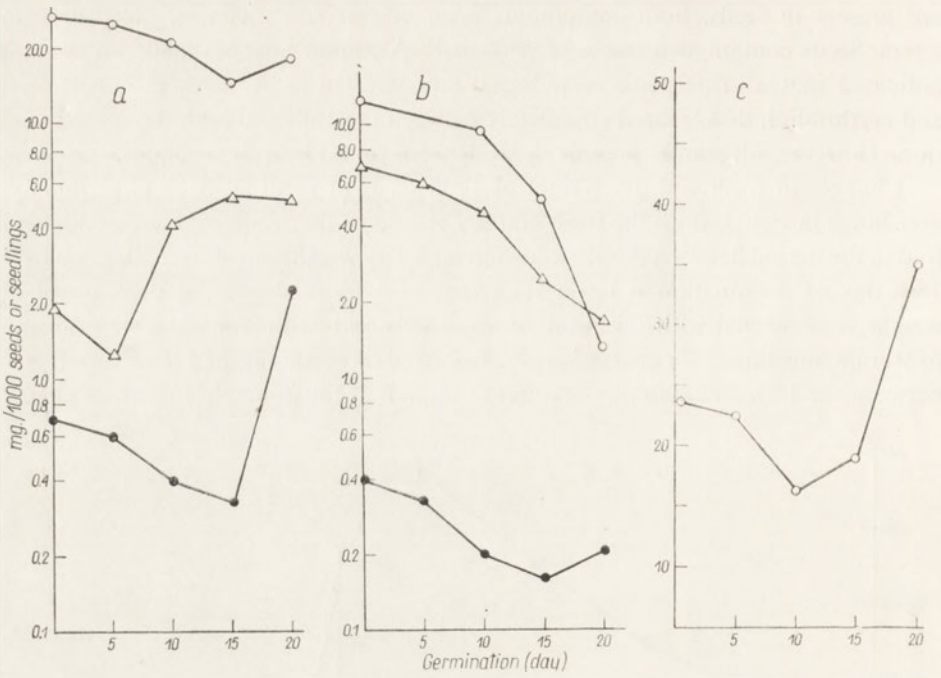

Fig. 5. Changes in the amount of sterols, triterpenic alcohols and oleanolic acid in *Calendula officinalis* during germination. *a*: (○), Free sterols; (△), sterol glycosides; (●), sterol esters; *b*: (○), free monols; (●), monol esters; (△), diol esters; *c*: (○), oleanolic acid. Note that, in Fig. 5c, a non-logarithmic scale is applied on the ordinate.

The data concerning the content of triterpenoid compounds are expressed in milligrams per 1000 seeds or seedlings. This manner of expression gives direct information whether the given compound is actually synthetized or metabolized.

Changes in the amount of free sterols, sterol glucosides and esters during germination of calendula are presented in Fig. 5. In seeds, sterols were present in 91% in free form, in 7% as glucosides and in 2% as esters. The amount of free and esterified sterols decreased during germination till the 15th day, and then an increase was observed. The amount of sterol glucosides in 5-day-old seedlings was lower than in the seeds but later, between the 5th and 10th day of vegetation, it increased. This could be due to glucosylation of sterols. The total amount of sterols in all fractions decreased till the 15th day of vegetation, whereas an increase was observed between the 15th and 20th day.

Changes in triterpenic alcohols are shown in Fig. 5a, b. In seedlings, free triterpenic diols were present in trace amounts. A steady decrease of free monols and diol esters was observed in the course of germination. A smaller decrease was found for the monol esters which constitute less than 10% of all monols.

The content of oleanolic acid decreased till the 10th day of germination and then increased till the end of the investigated period (Fig. 5c).

DISCUSSION

The presented results show that in calendula the amount of triterpenoid compounds decreases during germination. The triterpenoids are not discarded with seed shells which have been found to contain only insignificant amounts of sterols and contain no triterpenic alcohols or oleanolic acid. The decrease of triterpenoids till the 15th day of germination can be caused by their degradation or transformation into more oxidized derivatives.

Especially noteworthy is the demonstration in the seeds of considerable quantities of triterpenic alcohols found previously only in the flowers of calendula (Kasprzyk & Pyrek, 1968; Kasprzyk & Wojciechowski, 1969). They are transported most probably from the flowers to maturing seeds. During germination their content decreases steadily and, except β-amyrin and erythrodiol, they disappear between the 15th and 20th day. During this period the first leaves are formed and the metabolism characteristic of the mature plant begins to prevail. With increasing photosynthetic activity of the leaves, the dry weight of the plant rises considerably and enhanced biosynthesis of oleanolic acid, its precursors β-amyrin and erythrodiol, as well as of sterols is observed.

In our experiments the amount of triterpenoids was determined in whole seeds or seedlings. The studies of Kemp, Goad & Mercer (1967) on the changes in the amount of sterols in various morphological parts (root, stem, scutella and endosperm) of 4- to 13-day-old corn seedlings indicated that the biosynthetic processes occur specifically in distinct parts of the plant. In seeds the amount of free sterols was considerably higher than that of esterified sterols. During germination the amount of free Δ^5 and Δ^7 sterols increased significantly in stem and root, being unchanged in scutellum and endosperm. The amount of esterified sterols increased threefold in scutellum and remained constant in other parts of the plant, whereas the amount of Δ^7 sterol esters was unchanged in any of the morphological units.

It seems possible that also in calendula the synthesis of sterols and oleanolic acid occurs in stem and root, whereas degradation of these compounds and of triterpenic alcohols takes place in storage organ (cotyledons). The net result may be therefore a decreased content of sterols and oleanolic acid at the first stages of vegetation. It has been observed by Kasprzyk, Pyrek & Sliwowski (1967) that in 14-day-old seedlings of calendula, radioactivity of [l-^{14}C]acetate is incorporated into sterols. The results described in the present paper do not exclude the possibility that in some parts of the seedling the biosynthesis of sterols and oleanolic acid begins much earlier than on the 10th or 15th day of germination, whereas the degradation of triterpenoids proceeds simultaneously in other parts of the plant.

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ZMIANY TRÓJTERPENOIDÓW W KIEŁKUJĄCYCH NASIONACH *CALENDULA OFFICINALIS*

Streszczenie

Badano zmiany stężenia alkoholi trójterpenowych, kwasu oleanolowego oraz steroli wolnych, związanych estrowo i związanych glikozydowo, zachodzące w kiełkujących nasionach *Calendula officinalis*.

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ISOENZYMES OF ARGINASE IN RAT TISSUES

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1. The activity of arginase calculated per gram of fresh weight is in brain 1000-fold lower than in liver, and in kidney and submaxillary gland 30-fold lower. 2. The liver arginase showed one optimum at pH 9.5, whereas in other tissues studied two optima, at pH 7.5 and 9.5, were found. 3. By DEAE-cellulose chromatography, two arginase fractions were obtained from each tissue tested. The first fractions were eluted with tris buffer and could not be resolved by co-chromatography. The second fractions, which were eluted with different KCl concentrations, separated on co-chromatography. 4. The molecular weight determined by Sephadex G-100 gel-filtration was 120 000 for all separated isoenzymes, as well as for the unfractionated enzyme. The K_m values were virtually the same, ranging from 3.4 to 8.0 mM.

In our previous paper (Gąsiorowska, Porembska & Mochnacka, 1969) it has been demonstrated that many properties of the arginase (L-arginine amidinohydrolase, EC 3.5.3.1) from ox brain are identical with those of the arginase from liver of other ureotelic vertebrates. Moreover, two arginase isoenzymes have been found to occur both in ox brain and rat liver.

In the present work, a comparison has been made between the arginase from rat liver, and the arginase and isolated isoenzymes from rat kidney, submaxillary gland and brain, i.e. those tissues which do not take part in the process of ureogenesis.

MATERIALS AND METHODS

Reagents. They were the same as used previously (Gąsiorowska *et al.*, 1969).

Preparation of tissue extracts. White male rats, 3 - 4 months old (200 - 250 g.) were stunned and killed by decapitation. Liver, kidneys, brain and submaxillary glands were isolated, disintegrated and homogenized three times for 30 sec. et 1900 rev./min. with 3 vol. of 50 mM-$MnCl_2$ - 100 mM-KCl - 10 mM-tris-HCl buffer, pH 7.5, in a Teflon-glass Potter-Elvehjem homogenizer placed on ice. Then the homogenate was gently stirred at 0° for 15 min. and centrifuged at 5000 rev./min. for 15 min. at 4°. The supernatant was decanted, the sediment was again homo-

genized with the same amount of buffer as above, and the two supernatants were combined. The extracts from the particular tissues, when stored at -10° for about a week, did not lose their enzymic activity. Also in some experiments, in which the isolated organs were stored at -10°, no changes in activity were observed.

The extracts were used for experiments either directly or following 18-hr. dialysis against 10 mM-tris-HCl buffer, pH 7.5.

Chromatography on DEAE-cellulose. Tissue extracts were treated with ammonium sulphate to 0.9 saturation. After 1 hr. the sediment was collected by centrifugation, dissolved in 10 mM-$MnCl_2$ - 5 mM-tris-HCl buffer, pH 8.3, and dialysed overnight against the same buffer at 4°. Then the dialysis residue was cleared by centrifugation and applied to a DEAE-cellulose column (1 × 18 cm.) equilibrated with 5 mM-tris-HCl buffer, pH 8.3. The elution was carried out with 30 ml. of the equilibration buffer and then with a linear KCl concentration gradient (up to 0.3 M) in the same buffer. Fractions of 5 ml. were collected at a flow rate of 1.5 ml./min.

Subcellular fractions. The fractions from liver, kidney and submaxillary gland were prepared by the method of Hogeboom (1960) using 0.25 M-sucrose solution. For the brain, the procedure described by Whittaker, Michaelson & Kirkland (1964) using 0.32 M-sucrose, was employed. The particulate fractions after washing were suspended in 10 mM-tris-HCl buffer, pH 7.5, and frozen. For activity determination, they were thawed and gently homogenized.

Arginase assay. The reaction mixture contained in 1 ml.: 50 μmoles of L-arginine (adjusted to pH 7.5), 5 μmoles of $MnCl_2$, 50 μmoles of glycine buffer, pH 9.5, and enzyme preparation.

The amount of protein of the crude extract or purified enzyme preparation used per assay sample in separate experiments, is given in legends to Tables and Figures. The amounts of protein applied and time of incubation at 37° were within the range of linear rate of the reaction. The incubation time was generally 15 min., and for the brain extracts 30 min. The reaction was stopped by placing the samples for 10 min. in a boiling-water bath, and in the supernatant ornithine was determined by the method of Chinard (1952) or urea according to Archibald (1945) in the modification of Ratner (1955). Arginase activity was expressed as μmoles of ornithine or urea formed per minute.

Protein determination. The method of Lowry, Rosebrough, Farr & Randall (1951) was employed, with crystalline bovine serum albumin as standard. Protein in the effluent from the DEAE-cellulose column was determined spectrophotometrically by the method of Warburg & Christian (1941).

Molecular weight determination. This was performed according to Andrews (1965) by Sephadex G-100 gel-filtration.

RESULTS

Arginase activity in tissue extracts. The activity of arginase calculated per gram fresh tissue differed widely (Table 1). It was the highest in the liver, and was 30-fold lower in kidney and submaxillary gland, and 1000-fold lower in brain.

Table 1

The activity of arginase in rat tissues

The values are means from 3 experiments.

Tissue	Activity (μmoles ornithine/g. wet wt./min.)
Liver	330
Kidney	10
Submaxillary gland	9
Brain	0.28

One pH optimum at 9 - 9.5 was found for liver extract, and two optima, at pH 7.5 and 9.5, for extracts from kidney, brain and submaxillary gland (Fig. 1).

In neither of the tissues studied was arginase inactivated by 18-hr. dialysis of the extracts against 10 mM-tris buffer, pH 7.5 (without Mn^{2+} ions added), and in the case of liver extracts the specific activity following dialysis was even enhanced due

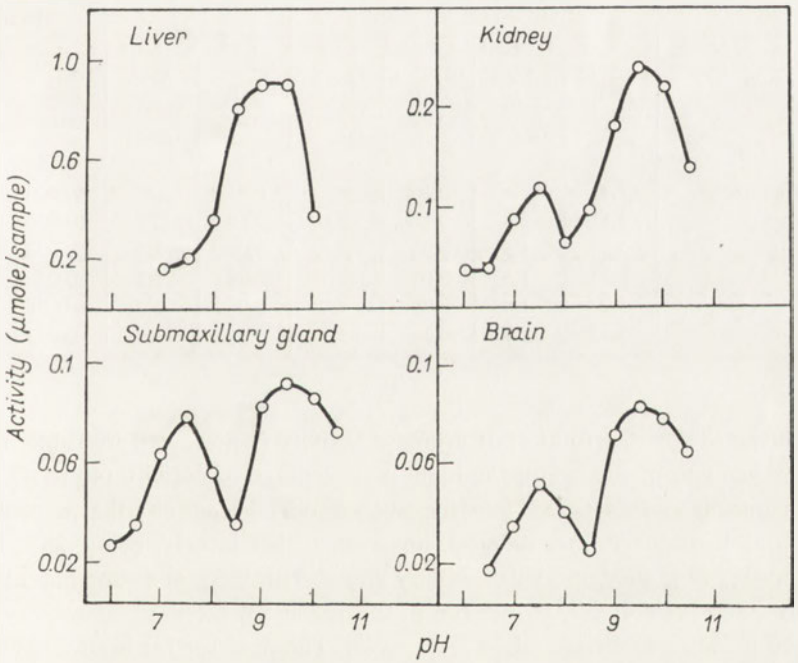

Fig. 1. The effect of pH on arginase activity in extracts from rat tissues. The incubation mixture contained in 1 ml.: 50 μmoles of L-arginine, 5 μmoles of $MnCl_2$, 50 μmoles of appropriate buffer, and tissue extract containing the following amounts of protein: liver, 0.02 mg.; kidney, 0.15 mg.; submaxillary gland, 0.05 mg.; brain, 1.0 mg. Incubation time was 15 min. For pH 6 - 8 tris-HCl, and for pH 9 - 10.5 glycine buffers were used.

to precipitation of inactive proteins. A 60-min. preincubation of the dialysed extracts with 5 mM-$MnCl_2$ at 37° activated only the liver arginase, whereas with extracts from other tissues no activation was observed, and sometimes the activity was somewhat decreased.

The effect of divalent cations on the activity of arginase in dialysed extracts is shown in Table 2. In all tissues studied, Mn^{2+} ion activated the enzyme to the greatest extent. Cd^{2+} ion activated more strongly the arginase from liver and kidney, and for Co^{2+} ion the reverse was true.

Table 2

The effect of divalent cations on arginase activity in dialysed extracts

The extracts were dialysed for 18 hr. against 10 mM-tris-HCl buffer, pH 7.5. Then the arginase activity was determined without or with chlorides of the indicated cations added. The assay sample contained in 1 ml.: 50 μmoles of arginine, 50 μmoles of glycine buffer, pH 9.5, 5 μmoles of the divalent cation, and tissue extract containing: liver 0.02 mg. of protein, kidney 0.15 mg., submaxillary gland 0.05 mg., brain 0.7 mg. The activity is expressed as μmoles of ornithine/mg. protein/min.

Cation added (mM)	Liver		Kidney		Submaxillary gland		Brain	
	Activity	Activation factor	Activity	Activation factor	Activity	Activation factor	Activity	Activation factor
None (control)	1.8		0.05		0.053		0.0033	
Mn^{2+}	4.1	2.3	0.1	2.0	0.123	2.3	0.0056	1.7
Mg^{2+}	2.34	1.3	0.06	1.2	0.08	1.5	0.0033	1.0
Co^{2+}	2.34	1.3	0.057	1.1	0.08	1.5	0.005	1.5
Cd^{2+}	3.2	1.8	0.088	1.7	0.066	1.2	0.0038	1.1
Ni^{2+}	3.12	1.7	0.06	1.2	0.053	1.0	0.0043	1.3

The intracellular distribution of arginase activity is presented in Table 3. In all tissues studied except the brain, the highest percentage of activity (40 to 47%) was found in nuclei, and 24 to 35% in the mitochondrial fraction; the percentage of activity found in microsomes differed, however, rather largely, being 28% in liver and about 9% in kidney and submaxillary gland. The 105 000 *g* supernatant contained only traces of activity. In the brain, the highest percentage of activity (49%) was found in mitochondria, and 34% in nuclei. The data for rat brain are in agreement with those for ox brain: 47% in mitochondria and 33% in nuclei (Gąsiorowska *et al.*, 1969) and are, may be, typical of mammalian brain. The distribution of arginase activity in rat liver is very similar to that reported by Mora, Martuscelli, Ortiz-Pineda & Soberón (1965): 40% in nuclei, 19% in mitochondria and 37% in microsomes.

Table 3

Percent distribution of arginase in subcellular fractions

The composition of the reaction mixture was as described in Methods. The amount of protein applied per assay sample was: liver 0.01 - 0.02 mg., kidney 0.13 - 0.35 mg., submaxillary gland 0.06 - 0.1 mg., brain 1.0 - 1.5 mg. The brain nuclei were separated at 1000 *g*. The results are expressed as percentages of the activity found in the whole homogenate.

Fraction	Arginase activity (%)			
	Liver	Kidney	Submax-illary gland	Brain
Homogenate	(100)	(100)	(100)	(100)
Nuclei (600 *g*, 15 min.)	40	45	47	34
Mitochondria (12 000 *g*. 15 min.)	24	35	35	49
Microsomes (105 000 *g*, 60 min.)	28	9	8	5
Supernatant	7	4	3	4

Fractionation on DEAE-cellulose of extracts from rat tissues. For each of the tissues studied, chromatography on DEAE-cellulose gave two peaks showing arginase activity (Fig. 2). Peak *I* was from every tissue eluted with 5 mM-tris-HCl buffer at nearly the same elution volume. The second peaks were eluted with a KCl concentration gradient, and appeared at 0.02 - 0.04 M-KCl for the submaxillary gland, at 0.06 - 0.11 M for liver, and at 0.13 - 0.16 M for kidney and brain. The active fractions, marked in the Figure by solid bars just above the horizontal axis, were pooled and used for further experiments. The distribution of activity between the two arginase peaks differed rather widely. In liver, peak *I* contained 70 - 80% of the activity recovered from the column, in kidney it represented 10 - 12%, in brain 70 - 75%, and in submaxillary gland 15 - 20% of the activity. The distribution of activity was highly reproducible; only in liver some variations were observed due to marked instability of the enzyme present in the second peak.

A comparison of the results for rat brain with those for ox brain (Gąsiorowska *et al.*, 1969) shows that in both cases two analogous arginase peaks were obtained, but the relative distribution of activity was quite dissimilar, in ox brain 30% of the activity being found in peak *I* and 70% in peak *II*. This difference could also be due to the instability of the enzyme present in peak *II* from rat-brain extract.

To check whether the differences observed in the appearance of peaks *II* were not due to interference with other proteins of the tissues studied, extracts from two different tissues were mixed and chromatographed together. Also in these conditions separate peaks *II* were obtained (Fig. 3).

The above results may indicate that in individual rat tissues two forms of arginase are present. One of them seems to be common to all tissues studied, and the second

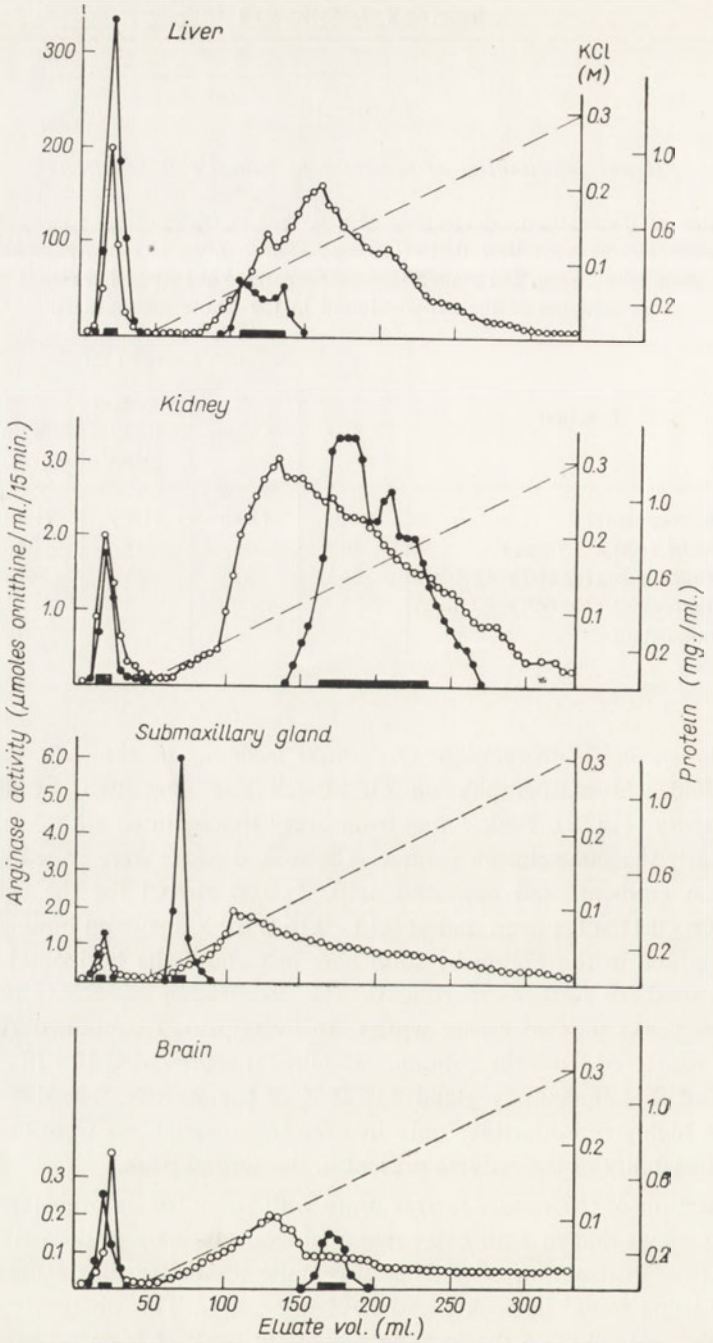

Fig. 2. Chromatography on DEAE-cellulose of extracts from rat tissues. The extract proteins precipitated with 0.9 ammonium sulphate sat. were dissolved in 10 mM-$MnCl_2$ - 5 mM-tris-HCl buffer, pH 8.3, dialysed against the same buffer and applied to the column (1 × 18 cm.). The amount of protein applied was: 80 mg. for liver, 160 mg. for kidney, 70 mg. for submaxillary gland, and 150 mg. for brain. The elution was first with 5 mM-tris-HCl buffer, pH 8.3, then with the same buffer with addition of a KCl concentration gradient up to 0.3 M. Fractions of 5 ml. were collected and (●), arginase activity and (○), protein were determined. The solid bars just above the horizontal axis show which fractions were pooled to be used in further experiments.

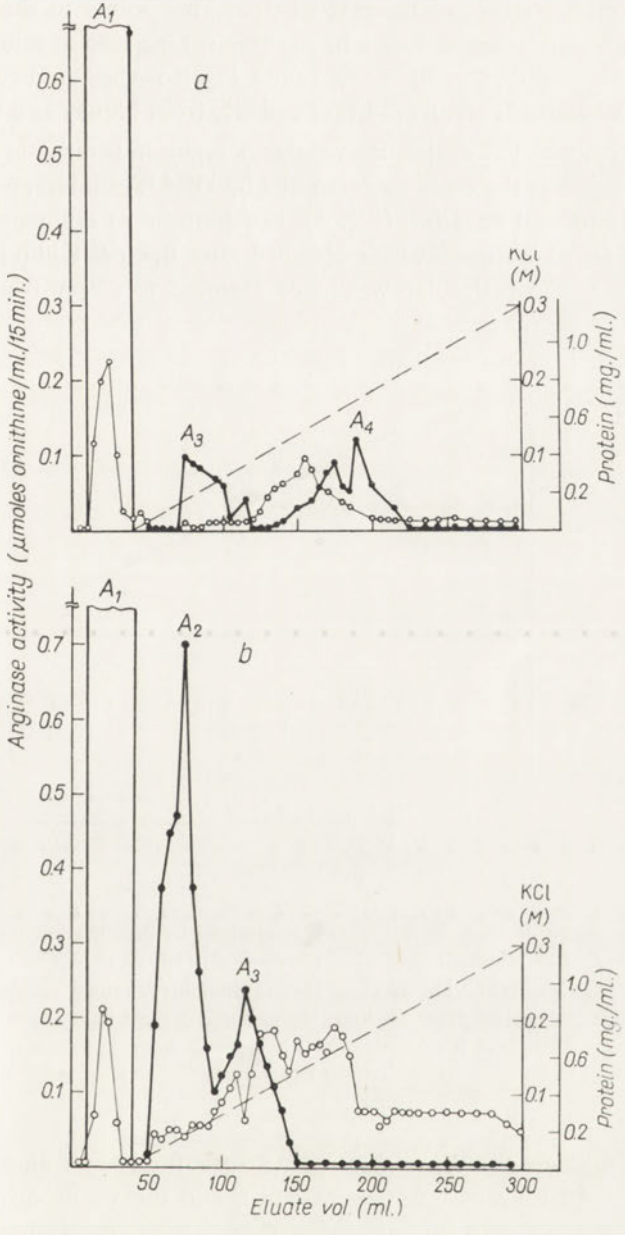

Fig. 3. The co-chromatography on DEAE-cellulose column of the mixture of: *a*, liver and kidney extracts, and *b*, liver and submaxillary gland extracts. For details see the legend to Fig. 2. (●), Arginase activity; (○), protein.

appears to be characteristic of the given tissue. This points to the occurrence of at least four different arginase forms in the rat. Taking into account their elution volume, they were called: fraction A_1, found in all tissues studied; fraction A_2, from submaxillary gland; A_3, from liver; and A_4, from kidney and brain.

Molecular weights. The molecular weight of arginase present in crude extracts from rat tissues and of the fractions separated by DEAE-cellulose, were determined by Sephadex G-100 gel filtration (Fig. 4). For purpose of comparison, a purified preparation of arginase from rat liver obtained after Bach & Killip (1961) was also used. In all cases the molecular weight of arginase was approximately 120 000.

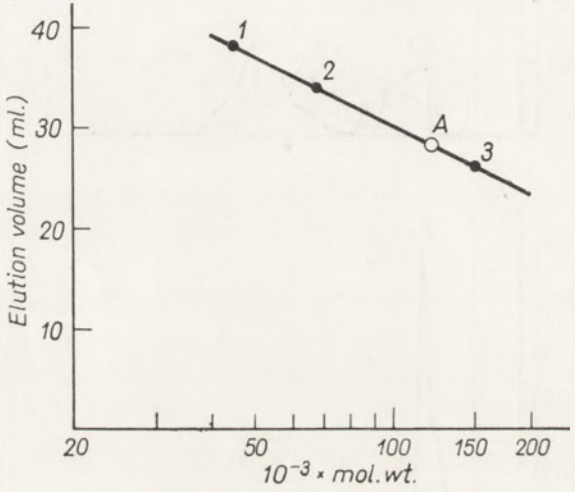

Fig. 4. Molecular weight of arginase isoenzymes determined by gel-filtration on Sephadex G-100 column (2×37 cm.). The eluting buffer was 100 mM-KCl - 10 mM-tris-HCl, pH 7.5. Stardard proteins (5 mg.): *1*, Egg albumin; *2*, human blood plasma albumin; *3*, human γ-globulin; *A* represents: the purified arginase preparation from rat liver, the arginase present in crude extracts of rat liver, kidney, submaxillary gland and brain, and the two arginase isoenzymes obtained from each of those tissues.

Stability of arginase fractions on storage. To the fractions *I* and *II* of arginase, $MnCl_2$ was added to a concentration of 5 mM, and the whole divided into three portions. Two of them were kept at -10°, and the activity determined, respectively, after two and eight days. In the third portion (control sample) the activity was assayed directly. The results presented in Table 4 indicate that the stability of the fractions from liver, submaxillary gland and brain were very similar. The activity of fraction *I* after 2 days at -10° was unchanged, and after 8 days only 10 - 15% of the activity was lost. Fractions *II* were unstable, especially that from the brain. On the other hand, the two fractions from kidney behaved in a quite different way, fraction *I* being unstable and fraction *II* exhibiting rather high stability.

Table 4

Stability of arginase fractions on storage at -10°

Samples of fractions isolated by DEAE-cellulose column chromatography and containing the indicated amount of protein/ml., were frozen and kept at -10°. After 2 or 8 days the samples were thawed and the activity determined as described in Methods. The results were calculated in μmoles of ornithine/mg. protein/min. In the Table, the relative activity as percentage of the activity determined directly (without freezing), is presented.

Tissue	Fraction	Protein in frozen sample (mg./ml.)	Activity (% of control) Day of storage	
			2	8
Liver	I	1.0	100	89
	II	0.5	24	10
Kidney	I	0.8	24	0
	II	0.9	100	85
Submaxillary gland	I	0.18	96	87
	II	0.08	64	26
Brain	I	0.2	100	85
	II	0.1	0	0

Kinetic data. The effect of substrate concentration on the initial reaction rate was assayed with 1.25 - 50 mM-arginine. The approximate K_m values determined from the plots of reciprocal velocity against reciprocal substrate concentration are presented in Table 5, and the Lineweaver-Burk plots for the isolated fractions in Fig. 5 (continuous lines). The K_m values were virtually the same ranging from 3.4 to 8.0 mM, these differences being within the limits of experimental error.

Table 5

Michaelis constants of arginase fractions from rat tissues

Tissue	K_m (mM)		
	Whole extract	Fraction *I*	Fraction *II*
Liver	5.0 ± 0.5	4.0	7.7
Kidney	4.0 ± 0.61	4.0	3.4
Submaxillary gland	3.4 ± 2.9	5.6	3.4
Brain	6.7 ± 0.7	8.0	—

In whole tissue extracts and in the isolated fractions, except liver fraction *I* and submaxillary gland fraction *II*, no substrate inhibition was observed. This was rather surprising as liver arginase is known to be inhibited by an excess of substrate and the same has been found for ox-brain arginase (Gąsiorowska *et al.*,

1969). This lack of substrate inhibition could perhaps be explained by the fact that in the present experiments with whole extracts and with the isolated fractions (with the exception of liver fraction *I* and submaxillary gland fraction *II*), arginine concentration in relation to the protein present in the sample was too small.

The effect of lysine and ornithine. These two basic amino acids, which are known inhibitors of liver arginase (Hunter & Downs, 1945), inhibited also the arginase in extracts from kidney, submaxillary gland and brain. At 5 mM concentration, lysine was found to be a stronger inhibitor for the kidney and brain, whereas ornithine for the liver and submaxillary gland enzymes. The inhibition by the two amino acids was competitive, except for kidney where it was of mixed type.

Lysine and ornithine had the same effect on the isolated fractions as they had on whole extracts. In Fig. 5 are presented the Lineweaver-Burk plots of arginine hydrolysis by the liver, kidney and submaxillary gland fractions, and by the whole extract from the brain, in the presence of the two inhibitors (dashed lines).

DISCUSSION

By DEAE-cellulose chromatography, two arginase fractions were found in rat kidney, submaxillary gland and brain, similar to those reported previously for rat liver and ox brain (Gąsiorowska *et al.*, 1969). The first fractions (A_1) were eluted with tris buffer at the same elution volume and, when mixed, could not be resolved by co-chromatography. It can be supposed that these four fractions are analogous but further studies, especially by immunoelectrophoretic methods, are required. The second fractions were eluted by increasing KCl concentrations in the following order: submaxillary gland (A_2), liver (A_3), kidney and brain (A_4). These fractions could be separated by co-chromatography, each retaining its specific elution characteristics. In this way it has been demonstrated that at least four arginase fractions occur in rat tissues. They can be considered as isoenzymes, and not as active arginase subunits since their molecular weights, determined by Sephadex G-100 gel-filtration, were the same as that of the unfractionated arginase present in tissue extracts, and amounted to 120 000. Affinity for the substrate was also practically the same in all isoenzymes and ranged from 3.4 to 8.0 mM. Preliminary studies on subcellular fractions of rat liver (unpublished data) indicate that mitochondria contain only fraction *I*, whereas both fraction *I* and *II* occur in nuclei.

Much smaller amounts of arginase were found in kidney, submaxillary gland, and especially in brain, than in the liver. This was to be expected since those tissues

Fig. 5. Lineweaver-Burk plots for arginase hydrolysis catalysed by the separated arginase fractions: (●), uninhibited, (△), in the presence of 5 mM-lysine, and (○), in the presence of 5 mM-ornithine. The reaction mixtures were as described in Methods and contained 1.25 - 50 mM-arginine. The amounts of protein per assay (1 ml.) were: liver fraction *I*, 0.0005 mg., fraction *II*, 0.001 mg.; kidney fraction *I*, 0.1 mg., fraction *II*, 0.1 mg.; submaxillary gland fraction *I*, 0.03 mg., fraction *II*, 0.012 mg. The whole brain extract used contained 1.5 mg. protein. The velocity, v, is expressed in μmoles urea/mg. protein/min.

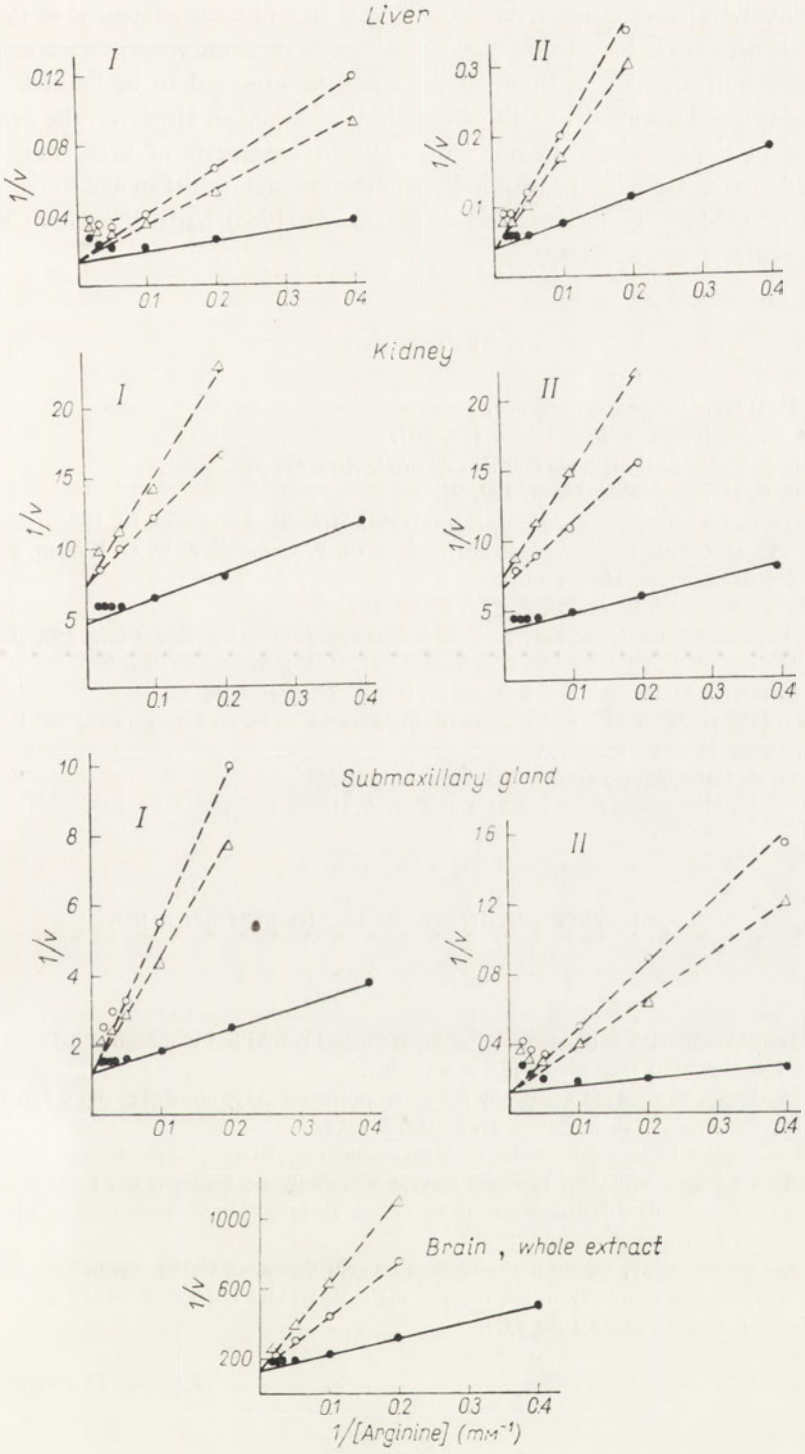

Fig. 5.

are not involved in ureogenesis, due either to the lack of some enzyme(s) of the ornithine cycle or, as in kidney, to the presence of one of these enzymes in trace amounts. The function of arginase in those tissues could be expected to be limited, like in tissues of uricotelic animals, to the catabolism of arginine. However, the arginases occurring in tissues which do not participate in biogenesis of urea, were found to differ from the arginases present in liver of the uricotelic chicken and lizard which, as reported by Mora, Tarrab, Martuscelli & Soberón (1965), had a K_m of 100 - 200 mM and molecular weight 270 000.

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IZOENZYMY ARGINAZY W TKANKACH SZCZURA

Streszczenie

1. Aktywność arginazy w przeliczeniu na gram świeżej tkanki jest w mózgu 1000 razy niższa, a w nerce i śliniance 30 razy niższa niż w wątrobie.

2. Stwierdzono, że arginaza z wątroby ma jedno optimum aktywności przy pH 9,5, natomiast enzym z innych tkanek — dwa optima przy pH 7,5 i 9,5.

3. We wszystkich badanych tkankach podczas chromatografii na DEAE-celulozie stwierdzono obecność dwóch frakcji arginazy. Pierwsze frakcje wymywały się buforem tris i nie rozdzielały się podczas ko-chromatografii; drugie frakcje wymywały się przy różnych stężeniach KCl i rozdzielały się podczas ko-chromatografii.

4. Ciężar cząsteczkowy, oznaczony sączeniem na żelu Sephadex G-100, wynosił dla enzymów obecnych w wyciągach tkankowych, jak również dla izoenzymów 120 000. Wartości K_m były tego samego rzędu i wynosiły od 3,4 do 8,0 mM.

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THE PREPARATION OF 2′-*O*-METHYLCYTIDINE-5′-MONO AND PYROPHOSPHATE, AND POLY-2′-*O*-METHYLCYTIDYLIC ACID

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1. 2′-*O*-Methylcytidine was converted on a preparative scale to 2′-*O*-methylcytidine-5′-phosphate with the aid of wheat shoot nucleoside phosphotransferase. 2. Chemical procedures were then employed to convert 2′-*O*-methylcytidine-5′-phosphate to the 5′-pyrophosphate. 3. The 5′-phosphate of 2′-*O*-methylcytidine was quantitatively dephosphorylated by the 5′-nucleotidase in *Viperia ammodytes* venom. 4. The 5′-pyrophosphate of 2′-*O*-methylcytidine proved to be a substrate of polynucleotide phosphorylase from *Micrococcus lysodeikticus* and *Escherichia coli*. 5. An essential requirement for polymerization was the replacement of normally employed Mg^{2+} cation by Mn^{2+}. 6. The poly-2′-*O*-methylcytidylic acid obtained exhibited sedimentation constants as high as 14.1 at neutral pH, and was hydrolysed to monomers by a mixture of snake venom phosphodiesterase, micrococcal nuclease and *E. coli* alkaline phosphatase. 7. Even in the presence of Mn^{2+}, thymidine-5′-pyrophosphate was inert towards polynucleotide phosphorylase, again confirming the specificity of the enzyme for the ribose moiety.

The occurrence of 2′-*O*-methyl (2′-*O*-Me)[1] ribonucleosides in nature is by no means uncommon. They are found in tRNA (Hall, 1964) and in the rRNA of bacterial (Nichols & Lane, 1967), yeast (Isaksson & Phillips, 1968) and mammalian (Wagner, Penman & Ingram, 1967; Lane & Tamaoki, 1969) cells. The rhapidosomes from a variety of flexibacterial strains have been reported to contain an RNA highly methylated on the ribose, in a manner claimed to be non-random, with the degree of methylation as high as 85% (Correll & Lewin, 1964; Correll, 1968); but independent confirmation of this has not yet been forthcoming.

[1] The following non-usual abbreviations are employed in this text: 2′-*O*-MeC, 2′-*O*-methylcytidine; 2′-*O*-MeCMP, 2′-*O*-methylcytidine-5′-phosphate; 2′-*O*-MeCDP, 2′-*O*-methylcytidine-5′-pyrophosphate; 2′-*O*-MeADP, 2′-*O*-methyladenosine-5′-pyrophosphate; poly-2′-*O*-MeC, poly-2′-*O*-methylcytidylic acid; poly-2′-*O*-MeA, poly-2′-*O*-methyladenylic acid; RNase, ribonuclease.

As might be anticipated, the 2′-*O*-methyl substituents confer resistance to ribonucleases which hydrolyse ribonucleotide linkages *via* 2′,3′-cyclic phosphate intermediates. It is consequently worthy of note that the presence has been reported in *Anacystis nidulans* of a "2′-*O*-Me RNase" which appears to be relatively specific for phosphodiester linkages containing a 2′-*O*-Me substituent (Norton & Roth, 1967). Confirmation of this finding is highly desirable, the more so in that phosphodiester bonds with a 2′-*O*-Me substituent are hydrolysed only with difficulty by snake venom phosphodiesterase (Gray & Lane, 1967) and micrococcal nuclease (Rottman & Henlein, 1968).

The 2′-hydroxyl in RNA has been implicated in various ways as a factor affecting polynucleotide structure, *via* hydrogen bonding to a base residue or to a neighbouring phosphate oxygen (e.g. see Ts'o, Rapaport & Bollum, 1966); but these hypotheses are largely speculative (Żmudzka, Bollum & Shugar, 1969), based on little or no direct experimental evidence. Blocking of the 2′-hydroxyls in either a natural or synthetic polyribonucleotide might be expected to provide some direct evidence for the role of the 2′-OH in the secondary structure of such polymers. One attempt at such a procedure was reported fairly recently, that of Knorre & Shamovsky (1967), who acetylated the 2′-OH groups in synthetic poly-rA and poly-rU and then examined the nature of the complexes formed by the acetylated chain of one of the polymers with the potentially complementary non-acetylated chain of the other. Their findings are, however, subject to considerable uncertainty because of the only partial nature of the acetylation reaction, and the lability of the *O*-acetyl groups, as well as their bulkiness, which might be expected to introduce appreciable steric or other hindrances.

It is clear that the preparation of homopolymers containing exclusively phosphodiester linkages with 2′-*O*-methyl substituents should make available more useful models for investigations on the physico-chemical and biological properties of the 2′-hydroxyl. Because of the specificity of polynucleotide phosphorylase with respect to the sugar residue, this appeared for some time to be a formidable task. The demonstration by Rottman & Henlein (1968) that 2′-*O*-MeADP is polymerized, albeit very slowly, by polynucleotide phosphorylase led us to attempt the synthesis of poly-2′-*O*-MeC.

The present communication describes the preparation of 2′-*O*-MeCMP, 2′-*O*-MeCDP and poly-2′-*O*-MeC with some of their properties. Additional properties of the latter are briefly described elsewhere (Żmudzka, Janion & Shugar, 1969).

RESULTS AND DISCUSSION

2′-O-*Methylcytidine*. The synthesis of this compound, which was kindly provided by Dr. C. B. Reese, has been described elsewhere (Martin, Reese & Stephenson, 1968). Its absorption spectrum at various pH values is shown in Fig. 1, based on

the molar extinction value measured in 0.1 N-HCl by Martin *et al.* (1968). Spectral titration at several wavelengths over the range pH 2 to 7 (not shown in the Figure) led to a pK_a for protonation of the ring $N_{(3)}$ nitrogen of 4.10. This is to be compared with the value for cytidine itself, under the same conditions (22°), of 4.1 (Fox & Shugar, 1952). Note also that the spectrum at pH 14 differs from that at

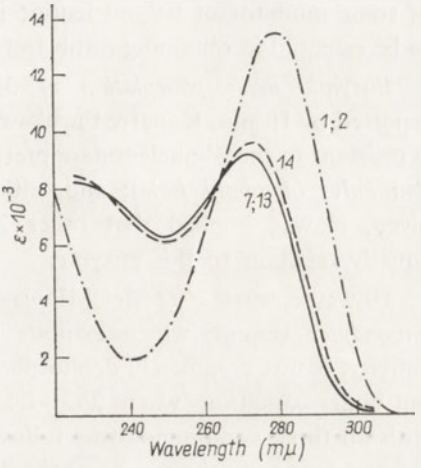

Fig. 1. Absorption spectrum of 2′-*O*-methylcytidine at various pH values indicated. Spectral titration at several wavelengths between pH 2 and 7 (not shown in Figure) gave a pK_a for protonation of the ring $N_{(3)}$ nitrogen of 4.09.

pH 7 - 13 due, as in the case of cytidine (Fox & Shugar, 1952) to dissociation of the (in this instance only the 3′- and/or 5′-) sugar hydroxyls.

Synthesis of 2′-O-methylcytidine-5′-mono- and 5′-pyrophosphate. Because of the limited amount of starting material, 2′-*O*-MeC, available, it was felt that phosphorylation by some enzymic procedure would be most advantageous.

It was, in fact, found in preliminary trials that 2′-*O*-MeC was a reasonably good substrate for wheat shoot nucleoside phosphotransferase; and, with the aid of the procedure described by Barner & Cohen (1959), using a large excess of *p*-nitrophenylphosphate as phosphate donor, 2′-*O*-MeCMP was routinely obtained in yields of the order of 50% with respect to the nucleoside. In all probability, even this yield could be improved upon. However, since isolation of the phosphorylated derivative by ascending chromatography on thick paper permitted the simultaneous recovery of unreacted nucleoside, no further attempts in this direction were made. Furthermore, while paper chromatography proved reasonably convenient for isolation of the product of phosphorylation, and recovery of unreacted nucleoside with the amount of substrate available to us (about 100 mg.), scaling up of the procedure to larger quantities would undoubtedly call for the use of column chromatography.

The eluted ammonium salt of 2′-*O*-MeCMP was then chemically converted to the morpholidate in about 90% yield by modification of the procedure of Moffatt & Khorana (1961).

The morpholidate, in turn, was converted to the 5′-pyrophosphate essentially as described by Moffatt & Khorana (1961). But separation of the 2′-*O*-MeCDP

product was carried out by column chromatography on DEAE-cellulose (carbonate form), which permitted of the simultaneous elimination from the potential substrate of inorganic phosphate, to give the trisodium salt of the 5′-pyrophosphate in about 75% yield with respect to the morpholidate. This was dissolved in water at a concentration of about 100 mg./ml. and precipitated by addition of 3 vol. ethanol in the cold. For reasons not entirely clear (but probably involving the removal of some inhibitor of polynucleotide phosphorylase), this precipitation step proved to be essential in obtaining a substrate which could be polymerized.

*Enzymic dephosphorylation of 2′-*O*-methylcytidine-5′-phosphate.* It has been reported by Honjo, Kanai & Furukawa (1964) that 2′-*O*-methyluridine-5′-phosphate is resistant to the 5′-nucleotidase present in bull semen and snake venom (*Agkistrodon halys blomhoffi Boie*); and, although no additional experimental data were given, it was implied that other 2′-*O*-methylribonucleoside-5′-phosphates were equally resistant to this enzyme.

However, when 2′-*O*-MeCMP was subjected to the action of an excess of *Viperia ammodytes* venom, which exhibits very low non-specific phosphomonoesterase activity, it was completely dephosphorylated, albeit at a slower rate than 5′-CMP, but under conditions where 2′(3′)-CMP was unaffected. The course of the reaction with all three compounds was followed by paper chromatography (Table 1). We are at a loss as to the source of the difference in our findings as compared to those of Honjo *et al.* (1964). It appears rather unlikely that we are dealing with some difference in enzyme specificities. Nonetheless, it may prove useful to examine the behaviour of 2′-*O*-MeCMP towards enzymes from other venoms.

*Polymerization of 2′-*O*-methylcytidine-5′-pyrophosphate.* Initial attempts to polymerize 2′-*O*-MeCDP by standard procedures, with the aid of polynucleotide phosphorylase preparations from *Micrococcus lysodeikticus* (Matthaei *et al.*, 1967) and *Escherichia coli* (Kimhi & Littauer, 1968), were unsuccessful despite the application

Table 1

R_F *values for some cytidine nucleotides*

Ascending chromatography on Whatman paper no. 1 and the following solvent systems (all proportions, by vol.) were applied: *A*, isopropanol - 1% ammonium sulphate (3:2); *B*, isobutyric acid - 1 M-NH_4OH - 0.1 M-EDTA, pH 8.2 (100:60:1.6); *C*, isopropanol - conc. NH_4OH - H_2O (7:1:2); *D*, ethanol - 1 M-ammonium acetate (5:2).

Compound	Solvent system			
	A	*B*	*C*	*D*
Cytidine	—	—	0.58	0.60
Cytidine-2′(3′)-phosphate	—	—	0.07	0.15
Cytidine-5′-phosphate	—	—	0.05	0.10
2′-*O*-Methylcytidine	0.87	0.88	0.85	0.83
2′-*O*-Methylcytidine-5′-phosphate	0.73	0.65	0.24	0.24
2′-*O*-Methylcytidine-5′-pyrophosphate	0.64	0.47	—	—

of a wide variation in reaction conditions. With the *M. lysodeikticus* enzyme, however, it was noted that polymerization of ADP was appreciably inhibited by an equimolar concentration of 2′-*O*-MeCDP, suggesting some affinity of the latter for the enzyme.

Polymerization was then examined on replacement of the Mg^{2+} cation, normally employed in this reaction, by Mn^{2+}. It was found in this way that the polymerization reaction, although as slow as that reported with 2′-*O*-MeADP as substrate (Rottman & Henlein, 1968), proceeded readily in the presence of Mn^{2+} with both enzymes. The use of this cation necessitated a small decrease in the pH of the incubation medium with the *M. lysodeikticus* enzyme to reduce its degree of oxidation. Reaction conditions were then selected by trial (see Experimental, below) to give polymerizations of the order of 60% of the substrate on incubation at 37° for 48 hr.

The incubation mixture was then brought to boiling to terminate the reaction and to precipitate some of the Mn^{2+} and protein, subjected to deproteinization by phenol treatment, isolated free from monomer (which was recovered) by passage through a Sephadex G-50 column, and finally dialysed exhaustively against salt, EDTA and water, and lyophilized. The overall yield of polymer, with respect to substrate, following lyophilization usually exceeded 55%.

Sedimentation constant and enzymic hydrolysis. The sedimentation constant, $S_{20,w}$, of poly-2′-*O*-MeC at approximately neutral pH in the presence of 0.15 M-Na^+ and 0.05 M-phosphate buffer (pH 6.5) or tris buffer (pH 8.0) varied from 9.0 to 14.1 for different preparations.

Treatment of the polymer with a mixture of snake venom phosphodiesterase, micrococcal nuclease and alkaline phosphatase at pH 9, as described by Rottman & Henlein (1968) for poly-2′-*O*-MeA resulted in slow hydrolysis to nucleosides, the accompanying hyperchromicity at the absorption maximum amounting to 37% (Fig. 2) as compared to 38% for poly-rC after RNase treatment. The modifications in absorption spectrum on hydrolysis, particularly in the region 285 - 300 mμ, are also similar to those accompanying enzymic hydrolysis of poly-rC.

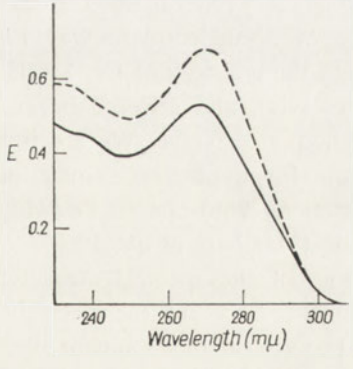

Fig. 2. Absorption spectrum of poly-2′-*O*-methylcytidylic acid at neutral pH (————) prior to and (— — — —) following enzymic hydrolysis to monomers. Hydrolysis conditions: 0.1 M-tris buffer, pH 9.0, and 0.015 M-$MgCl_2$, with 0.15 mg. snake venom phosphodiesterase, 0.015 mg. micrococcal nuclease and 0.1 mg. *E. coli* alkaline phosphatase (cf. Rottman & Henlein, 1968). Incubation for 20 hr. at 37°. Control contained all components with exception of polymer.

Specificity of polynucleotide phosphorylase. This enzyme has been generally regarded as highly specific for the ribose moiety (Grunberg-Manago, 1963). But the

fact that both 2′-*O*-MeADP (Rottman & Henlein, 1968) and 2′-*O*-MeCDP are suitable substrates might appear, at first sight, to raise some doubts as to the validity of this conclusion. Furthermore, it should be recalled that, on replacement of Mg^{2+} by Mn^{2+}, DNA polymerase will incorporate *ribo*nucleoside triphosphates into a growing chain (Berg, Fancher & Chamberlin, 1963), although no interpretation for this striking observation has been forthcoming.

In view of the foregoing, thymidine-5′-pyrophosphate was examined as a potential substrate with both the *M. lysodeikticus* and *E. coli* polynucleotide phosphorylases, in the presence of Mn^{2+}. The results were entirely negative, in agreement with the specificity of the enzyme towards the ribose moiety.

It would clearly be desirable to examine in greater detail the cation requirements and the kinetics of the polymerization reaction for this enzyme. Pertinent to this is the previously reported observation that polymerization of rGDP to poly-rG is apparently more effective in the presence of Mn^{2+} than Mg^{2+} (Thang, Graffe & Grunberg-Manago, 1965).

The specificity of polynucleotide phosphorylase with respect to the base residue is, by contrast, fairly broad. There is, on the other hand, at least one puzzling exception, e.g. 5-ethyluridine-5′-pyrophosphate is slowly polymerized, but in good yield, by the enzyme from *E. coli*, but not by that from either *M. lysodeikticus* or *Azotobacter vinelandii* (Świerkowski & Shugar, 1969). A similar situation appears to prevail with regard to 5,6-dihydrouridine-5′-pyrophosphate.

EXPERIMENTAL

2′-*O*-Methylcytidine (Martin *et al.*, 1968) was a gift from Dr. C. B. Reese. 2′(3′)-CMP and 5′-CMP were products of Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The sodium salt of *p*-nitrophenylphosphate was a B.D.H. (Poole, England) product. Snake venom phosphodiesterase, micrococcal nuclease and *E. coli* alkaline phosphatase were obtained from Worthington (Freehold, N. J., U.S.A.). The source of 5′-nucleotidase was *Viperia ammodytes* lyophilized venom, provided through the courtesy of Dr. Maria Drakulic (Institute of Nuclear Studies, Zagreb, Yougoslavia), and selected because of its extremely low content of non-specific phosphomonoesterase. We are indebted to Dr. U. Z. Littauer of the Weizmann Institute for a purified sample of *E. coli* polynucleotide phosphorylase (Kimhi & Littauer, 1968) and to Dr. H. Matthaei for a sample of the *M. lysodeikticus* enzyme (Matthaei *et al.*, 1967).

Most of the spectral measurements were performed with a Unicam SP-500 instrument. Some of the spectral curves, e.g. for titration of 2′-*O*-methylcytidine (Fig. 1), were run on a Unicam SP-800 recording instrument. Sedimentation constants were obtained with the aid of a Beckman Model E ultracentrifuge with ultraviolet optics. For control and measurement of pH, a Radiometer PHM-24 instrument with a semi-micro glass electrode was utilized.

2′-O-Methylcytidine-5′-phosphate. The nucleoside phosphotransferase enzyme was prepared essentially as described by Barner & Cohen (1959). Young (5 - 7

days) wheat shoots, derived from seeds cultivated in the dark, were chopped up and a suspension of about 1 g. in 10 ml. water homogenized at high speed. The homogenate was centrifuged to give a clear solution, which served as the source of enzyme. The protein content of the enzyme solution was determined spectrally according to Kalckar (1947). This preparation is very stable and exhibited no loss in activity after storage at -40° for more than a year.

Phosphorylation of 2′-*O*-MeC was carried out portionwise in a medium which contained, in a total volume of 5.5 ml.: 30 mg. (about 0.115 m-mole) 2′-*O*-MeC; 670 mg. (2 m-moles) *p*-nitrophenylphosphate; 2 ml. 0.2 M-acetate buffer, pH 5.2; 2.5 ml. enzyme solution (protein content 6.8 mg.). Incubation was usually for 18 hr. at 37°. The progress of the reaction was followed by removal at various time intervals of 10 µl. aliquots, which were subjected to ascending chromatography on Whatman paper no. 1 with solvent system *C* of Table 1. In this system the R_F values of 2′-*O*-MeC and 2′-*O*-MeCMP were 0.85 and 0.25, and the corresponding values for *p*-nitrophenylphosphate and *p*-nitrophenol 0.61 and 1.0. The nucleoside and nucleotide spots, following location under a dark UV lamp, were eluted with equal volumes of 0.1 N-HCl. Assuming the extinction coefficients of the nucleoside and nucleotide to be identical, the reaction yield under the above conditions was usually about 45 - 50%.

For preparative purposes, the entire reaction volume was streaked on to Whatman paper no. 3*MM*, about 1 ml. per 100 cm. Following ascending chromatography with the same solvent for 48 hr. at 18°, the strips containing the nucleoside and nucleotide were cut out and extracted with water. The unreacted nucleoside was collected and used in a subsequent phosphorylation reaction. The solution of the ammonium salt of the eluted mononucleotide was finally lyophilized.

Susceptibility of 2′-O-*methylcytidine-5′-phosphate to 5′-nucleotidase.* The source of 5′-nucleotidase was the lyophilized *Viperia ammodytes* venom, the non-specific phosphomonoesterase level of which is very low. The incubation medium contained 5 mg./ml. substrate and 5 mg./ml. snake venom in 0.05 M-borate buffer, pH 8.9, and 0.01 M-$MgCl_2$. Under these conditions, 2′-*O*-MeCMP was completely dephosphorylated in 18 hr. at 37°. At 22°, dephosphorylation was 60% complete in 18 hr., during which period 5′-CMP was completely dephosphorylated, whereas 2′(3′)-CMP was not noticeably affected. Dephosphorylation was followed in all instances by paper chromatography, the R_F values of the various relevant compounds being presented in Table 1.

2′-O-Methylcytidine-5′-phosphoromorpholidate. The ammonium salt of 2′-*O*-MeCMP, 0.21 m-mole (estimated spectrally), was dissolved in 5 ml. water and treated with 100 mg. Dowex 50W X8 (H^+), 200/400 mesh, the total capacity of which was 5 mEq/g. Transformation of the ammonium salt of the nucleotide to the free acid resulted in some turbidity, which was removed by addition of water, following which the resin was filtered off. A spectral control showed that all the nucleotide was in the filtrate. The latter was brought to dryness, 70 µl. morpholine (freshly distilled) added to the residue, and the whole dissolved in 2 ml. water and

2 ml. *tert.*-butanol. The solution was heated under reflux, and 165 mg. of dicyclohexylcarbodiimide in 3 ml. *tert.*-butanol added over a period of 4 hr., following which heating was continued for an additional 4 hr. A chromatographic control, using solvent system *C*, exhibited a single spot corresponding to the morpholidate and disappearance of 2′-*O*-MeCMP. The solution was brought to room temperature, 5 ml. water added, the resulting precipitate filtered off, and the filtrate brought to small volume and extracted three times with ether. Spectral assay demonstrated the presence of 0.19 m-mole (90% yield) of the morpholidate of 2′-*O*-MeCMP.

2′-O-Methylcytidine-5′-pyrophosphate. 2′-*O*-Methylcytidine-5′-phosphoromorpholidate (4-morpholine *N,N′*-dicyclohexylcarboxamidine salt), 0.19 m-mole, was dried by three distillations from 3-ml. portions of anhydrous pyridine. In a separate container, 0.6 m-mole (41 µl.) of 85% orthophosphoric acid and 0.6 m-mole (143 µl.) of distilled tri-*n*-butylamine was dried by four distillations from 3-ml. portions of anhydrous pyridine, the residue dissolved in 2 ml. anhydrous pyridine, combined with the solution of the morpholidate, and the mixture left at room temperature for 4 days. Chromatography with solvent system *C* demonstrated the disappearance of the morpholidate, the presence of traces of 2′-*O*-MeCMP, and a strongly intense spot corresponding to 2′-*O*-MeCDP.

The pyridine solution was brought to dryness under reduced pressure and traces of pyridine removed by distillation from water. The residue was dissolved in 30 ml. water, the cyclohexylurea precipitate filtered off, and the solution deposited on a 2.9×23 cm. column of DEAE-cellulose (carbonate form, with a total capacity of 0.7 mEq/g. dry mass). The column was first washed with 1 litre water, and elution then conducted with a linear concentration gradient of triethylammonium bicarbonate (formed from 1.3 l. of a 0.005 M solution and 1.3 l. of a 0.3 M solution). Fractions of 15 - 17 ml. were collected at intervals of 4 - 5 min. Traces of the morpholidate and the 5′-monophosphate were removed at buffer concentrations of 0.075 M to 0.11 M, and the 5′-pyrophosphate in the range 0.11 M to 0.175 M. Inorganic phosphate, determined by the method of Fiske & Subbarow (1925), was partially eluted along with the 5′-monophosphate, but disappeared before the 5′-pyrophosphate peak was reached. The fractions containing the 5′-pyrophosphate (0.14 m-mole by spectral estimation) were pooled, and brought to dryness under reduced pressure at 38°. Triethylammonium bicarbonate was then removed by three distillations from 20 ml. portions of methanol. The residue was dissolved in 3 ml. methanol, to which was added 0.7 ml. of 1.0 M-NaI in acetone, and 50 ml. acetone added to give a precipitate. The precipitate was centrifuged off and washed with three 20-ml. portions of acetone. The dried precipitate amounted to 70 mg., which corresponds to 0.14 m-mole as the trisodium salt with 2 molecules of water of hydration (74% yield with respect to morpholidate). The product was chromatographically homogeneous with solvents *B* and *C*.

It should be emphasized that the product was active as a substrate for polynucleotide phosphorylase *only* after ethanol precipitation from a concentrated aqueous solution. To 20 mg. of 2′-*O*-MeCDP dissolved in 200 µl. water, and centri-

fuged to remove a slight turbidity, was added about 3 vol. ethanol. The product was collected by centrifugation (yield ~ 90%).

Poly-2′-O-methylcytidylic acid. For the polymerization on a larger scale the *M. lysodeikticus* enzyme was used. The course of polymerization was followed by paper chromatography, based on disappearance of substrate, using solvents *A* and *B*; and by appearance of an absorbing product at the start.

Application of optimal polymerization conditions suitable for a variety of ribonucleoside-5′-pyrophosphates gave completely negative results with 2′-*O*-MeCDP. No improvement was forthcoming from a variation in pH over the range 8.5 to 10, a twofold increase or up to a tenfold decrease in concentration of substrate, or a twofold increase or up to a tenfold decrease in Mg^{2+} concentration. Variations in incubation temperature were likewise without effect. Only on replacement of Mg^{2+} by Mn^{2+} was any polymerization observed. The use of this cation necessitated a small decrease in the pH of the incubation medium to reduce its degree of oxidation. After a number of trials, the incubation medium settled on was as follows: 0.7 μmole 2′-*O*-MeCDP; 30 μl. 0.5 M-tris buffer, pH 8.5; 40 μl. 0.025 M-$MnSO_4$; 4 μl. 0.01 M-Na-EDTA; 10 μl. 0.01 M-NaH_3; 10 μl. of the *M. lysodeikticus* enzyme; and water to a total volume of 100 μl. Incubation was at 37°, and up to 60% substrate was incorporated into polymer in periods ranging from 24 to 48 hr.

The reaction was terminated by immersion of the reaction vessel in a boiling-water bath for 2 min., which resulted also in precipitation of some protein and manganese. This was removed by centrifugation and washing of the precipitate. The polymer solution was then subjected to three deproteinizations with freshly-distilled, neutralized phenol, and the aqueous phase reduced in volume under low pressure at 25° so that the polymer concentration was about 3 - 4 mg./ml. This was deposited on a 2.1 × 60 cm. column of Sephadex G-50 (coarse) and the polymer fraction eluted with 0.05 M-triethylammonium bicarbonate and brought to dryness. It was then dissolved in water and dialysed successively against decreasing NaCl concentrations, EDTA and finally water, then lyophilized. The final yield, on a molar basis with respect to substrate, was 55% from an incubation mixture which included 10 mg. substrate.

Enzymic degradation of poly-2′-*O*-MeC was carried out as described in the legend to Fig. 2.

We are deeply indebted to Dr. C. B. Reese, whose gift of 2′-*O*-methylcytidine made this investigation possible; to Dr. H. Matthaei and Dr. U. Z. Littauer for the samples of polynucleotide phosphorylase from *M. lysodeikticus* and *E. coli*, respectively, and to Mr. H. Sierakowski for measurements of sedimentation constants. This study profited from the support of the Wellcome Trust, the World Health Organisation, and the Agricultural Research Service, U.S. Dept. of Agriculture (UR-E21-(32)-30).

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OTRZYMANIE 5′-MONO I PIROFOSFORANU 2′-*O*-METYLOCYTYDYNY ORAZ KWASU POLI-2′-*O*-METYLOCYTYDYLOWEGO

Streszczenie

1. Poprzez enzymatyczną fosforylację 2′-*O*-metylocytydyny fosfotransferazą nukleozydową z siewek pszenicy otrzymano 5′-fosforan 2′-*O*-metylocytydyny.

2. Następnie na drodze przekształceń chemicznych z 5′-fosforanu 2′-*O*-metylocytydyny otrzymano 5′-pirofosforan 2′-*O*-metylocytydyny.

3. 5′-Fosforan 2′-*O*-metylocytydyny jest ilościowo defosforylowany przez 5′-nukleotydazę *Viperia ammodytes*.

4. Stwierdzono, że 5′-pirofosforan 2′-*O*-metylocytydyny jest substratem dla fosforylazy polinukleotydowej z *Micrococcus lysodeikticus* i *Escherichia coli*.

5. Istotnym warunkiem polimeryzacji było zastąpienie jonów Mg^{2+}, jonami Mn^{2+}.

6. Otrzymany kwas 2′-*O*-metylocytydylowy posiadał w pH obojętnym wysoką stałą sedymentacji — 14.1, a pod wpływem mieszaniny fosfodiesterazy jadu węża, nukleazy mikrokokalnej i fosfatazy alkalicznej *E. coli*, był hydrolizowany do nukleozydów.

7. 5′-Pirofosforan tymidyny nawet w obecności Mn^{2+} nie był substratem dla fosforylazy polinukleotydowej, co potwierdza raz jeszcze specyficzność enzymu dla części cukrowej.

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STABILIZATION OF ISOLEUCYL-tRNA SYNTHETASE FROM YELLOW LUPIN SEEDS BY TRANSFER RNA

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Isoleucyl-tRNA synthetase from yellow lupin seeds is protected against thermal inactivation at 50° by lupin tRNA. The stabilization is due to isoleucine-specific tRNA; other tRNA species are inactive. Heterologous tRNA's, from yeast and *E. coli* cells, stabilize the enzyme to a much smaller extent. The presence of Mg^{2+}, Ca^{2+} ions, or even Sr^{2+} which is inactive in the tRNA aminoacylation reaction, is required for the protective action of $tRNA^{Ile}$. This may indicate that the divalent cations have a stabilizing effect on tertiary conformation of tRNA, indispensable for formation of tRNA - enzyme complexes.

One of the crucial reactions in protein synthesis is a highly specific, two-stage transfer of an amino acid to RNA, catalysed by an aminoacyl-tRNA[1] synthetase (amino acid : tRNA ligase (AMP)). This key process has not been fully elucidated and therefore data concerning all interactions of these enzymes with their substrates and the formation of intermediates may contribute to better understanding of this reaction.

Several authors have reported on the isolation of enzyme - aminoacyl - AMP complexes (Allende, Mora, Gatica & Allende, 1965; Lagerkvist, Rymo & Waldenström, 1966; Chousterman, Sorino, Stone & Chapeville, 1968; Hirsch, 1968; Rouget & Chapeville, 1968), the protein components of which appear to the stabilized against heat inactivation (Chuang, Atherly & Bell, 1967; Rouget & Chapeville, 1968). Also the formation of synthetase complexes with corresponding tRNA's is well documented (Lagerkvist *et al.*, 1966; Okamoto & Kawade, 1967; Yarus & Berg, 1967; Seifert, Nass & Zilling, 1968). However, in the one so far investigated case, yeast seryl-tRNA synthetase was not protected against thermal inactivation by its specific tRNA (Makman & Cantoni, 1966). As the phenomenon of stabilization of enzymes by their substrates is very often observed, it might be supposed that some of the synthetases will be stable at higher temperatures in the presence of their specific tRNA's.

[1] Abbreviations used: rRNA, ribosomal RNA; tRNA, transfer RNA; $tRNA^{Ile}$, isoleucine-specific tRNA; $tRNA^{Lys}$, lysine-specific tRNA; GSH, reduced glutathione.

In our studies we have found that thermal inactivation of isoleucyl-tRNA synthetase of plant origin is partly overcome by $tRNA^{Ile}$. The factors affecting this protection are described in the present report.

MATERIALS AND METHODS

Material

Uniformly labelled L-[^{14}C]isoleucine, 79.3 mc/m-mole, L-[^{14}C]leucine, 78 mc/m-mole, and L-[^{14}C]lysine, 88.5 mc/m-mole, were obtained from the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia); DEAE-cellulose DE 11 was a product of W. & E. Balston Ltd. (Maidstone, Kent, England); ATP was from Carl Roth (Karlsruhe, West Germany) and was neutralized with tris before use; 2-mercaptoethanol was from Koch-Light Lab. Ltd. (Colnbrook, Bucks., England), glutathione from Sigma Chem. Co. (St. Louis, Mo., U.S.A.); tris(hydroxymethyl)aminomethane was a product of British Drug Houses Ltd. (Poole, Dorset, England); 2,5-diphenyloxazole (PPO) was from Reanal (Budapest, Hungary) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) from Calbiochem. (Los Angeles, Calif., U.S.A.).

tRNA from *Escherichia coli* B and tRNA from yeast were purchased from Schwarz Bioresearch Inc. (Orangeburg, N.Y., U.S.A.). Yellow lupin seed tRNA was isolated as described by Legocki, Szymkowiak, Wiśniewski & Pawełkiewicz (to be published); the crude preparation extracted with NaCl contained 4% of isoleucine-specific tRNA, and after DEAE-Sephadex column chromatography, about 20%. The highly purified preparations contained, respectively, 83% of $tRNA^{Ile}_{II}$ and 75% of $tRNA^{Lys}$.

Ribosomal RNA was the first fraction of yellow lupin seed RNA from Sephadex G-200 column (Legocki, Szymkowiak, Pech & Pawełkiewicz, 1967).

Methods

*Isolation of isoleucyl-*tRNA *synthetase from yellow lupin seeds.* All operations were carried out at 0 - 4°. Yellow lupin meal, 67 g., was treated for 30 min. with 200 ml. of 0.1 M-potassium-phosphate buffer, pH 7.6, containing 0.4 M-sucrose, 1 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol. The slurry was pressed through cheesecloth, adjusted with diluted KOH solution to pH 7.4, and centrifuged for 20 min. at 60 000 *g*. To the supernatant containing about 6 m-units of enzyme activity/mg. of protein, was added 20% solution of streptomycin sulphate neutralized with K_2HPO_4 solution. To 20 vol. of the supernatant, 1 vol. of streptomycin sulphate solution was added. After 30 min., to each 100 ml. 30 ml. of saturated, neutral ammonium sulphate solution was added and after a further 30 min. the sediment was centrifuged off for 15 min. at 60 000 *g*. To the clear supernatant, saturated ammonium sulphate solution was again added at a ratio 2:1 (v/v) and the sediment was collected by centrifugation. Then it was dissolved in 10 ml. of buffer *A* (20 mM-potassium-phosphate buffer, pH 7.3, containing 40 mM-KCl, 1 mM-$MgCl_2$ and

1 mM-2-mercaptoethanol) and the obtained solution was dialysed against the same buffer for 4 hr. At this point the activity of isoleucyl-tRNA synthetase usually increased to 45 m-units/mg. of protein. For further purification, the enzyme solution was applied to a DEAE-cellulose column (5×4.5 cm.) equilibrated with buffer *A*, and washed with 90 ml. of the same buffer. The enzyme was eluted with 125 ml. of buffer *A* containing additionally 0.16 M-KCl. The collected fraction was treated with solid ammonium sulphate to the final saturation of 0.8, and the precipitated protein was kept in this solution at -20° for several weeks without loss of activity. Before use, the protein was dissolved in a small volume of 20 mM-potassium-phosphate buffer, pH 7.3, containing 1 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol, and dialysed against this solution overnight. At this stage the activity of the enzyme amounted to about 250 m-units/mg. of protein, being 40 times that of crude extracts. The preparation was free from ribonucleases, as checked by Tanaka's (1966) method, although it contained leucyl- and lysyl-tRNA synthetase activities.

*Assay of isoleucyl-*tRNA *synthetase activity.* The formation of [^{14}C]isoleucyl-tRNA was measured in a reaction mixture containing in 0.5 ml.: 50 μmoles of tris-HCl buffer, pH 7.5; 5 μmoles of $MgCl_2$, 5 μmoles of KCl, 2 μmoles of ATP, 1 μmole of GSH, 6 mμmoles of [U-^{14}C]isoleucine (0.48 μc), 1 mg. of tRNA and 0.1 - 1.6 mg. of enzyme protein. The incubation was carried out for 20 min. at 37°. Then 0.9 ml. of 10% trichloroacetic acid solution containing non-radioactive isoleucine (2 mg./ml.) was added at 0°. After 1 hr. the sediment was spun down, washed twice with 2 ml. of cold ethanol and once with ethyl ether, dried and dissolved in 0.2 ml. of concentrated formic acid. Then 4 ml. of Bray (1960) liquid scintillator was added, and the radioactivity measured in the scintillation counter SE 2 (Biuro Urządzeń Techniki Jądrowej, Warszawa, Poland) with the efficiency of 50%. Blank values were obtained either by adding boiled enzyme solution to the reaction mixture, or by omitting tRNA.

One unit of enzyme activity was defined as that amount which resulted in formation of 1 mμmole of isoleucyl-tRNA under standard conditions of the assay.

*Standard assay of changes in isoleucyl-*tRNA *synthetase activity during thermal inactivation.* Heating of enzyme was carried out in a reaction mixture containing in 80 μl.: 10 μmoles of tris-HCl buffer, pH 7.5, 1 μmole of $MgCl_2$, 1 μmole of KCl, 0.2 mg. of tRNA, 1 μmole of 2-mercaptoethanol, and 0.1 mg. of enzyme protein. Before the inactivation the mixture was always preincubated for 5 min. at 30° and then heated for a further 5 min. at 50°. The control sample was not heated.

To determine the remaining activity, to the cooled sample 6 mμmoles of [U-^{14}C]-isoleucine (0.48 μc) and 0.5 μmole of ATP were added in a volume of 20 μl. and the whole incubated for 20 min. at 37°. Then 80 μl. portion of the reaction mixture was applied to Whatman 3 MM paper disc (⌀ 25 mm.) and the disc was washed three times, for 15 min. each, in 5% trichloroacetic acid solution, once in ethanol - ether mixture (1:1, v/v) and finally in ether. The dried paper was placed in a vial containing 3 ml. of liquid scintillator (4 g. of PPO, 0.1 g. of POPOP in 1 litre of toluene) and counted in the scintillation counter. Additional details or modifications are described in legends to Tables.

RESULTS

*Thermal inactivation of isoleucyl-*tRNA *synthetase.* Heat treatment of enzyme at 50° completely suppressed the activity within 3 min. (Table 1). However, the enzyme appeared to be relatively stable in the presence of a crude tRNA preparation, more than 40% of the initial activity remaining even after 9 min. of heating. The action of tRNA appeared to be rather specific as rRNA had no protective effect. Neither did isoleucine with ATP protect the enzyme, which suggests that the synthetase was not stabilized in a complex of enzyme - aminoacyl - AMP under the conditions used. However, some stabilizing effect of ATP could be observed at 45° (Fig. 1).

The stabilization of isoleucyl-tRNA synthetase by tRNA was not a property common to all lupin synthetases. The leucine- and lysine specific synthetases, present in the partially purified preparation, were not stabilized by tRNA.

*Specific stabilization of isoleucyl-*tRNA *synthetase by* $tRNA^{Ile}$. The stabilization of isoleucyl-tRNA synthetase raised the question whether the effect of the crude tRNA preparation is due to its isoleucine-specific component(s). Therefore the crude lupin tRNA preparation was fractionated by chromatography on DEAE-cellulose at 72° according to Legocki, Szymkowiak & Pawełkiewicz (1968) and three fractions (Fig. 2), namely the 53rd, 59th and 63rd containing, respectively, 14, 2 and

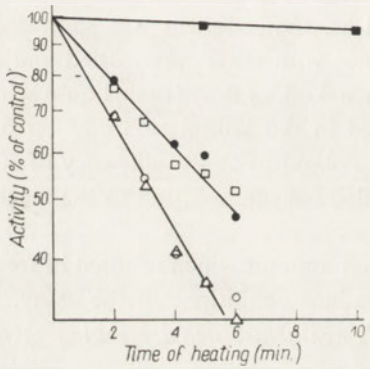

Fig. 1

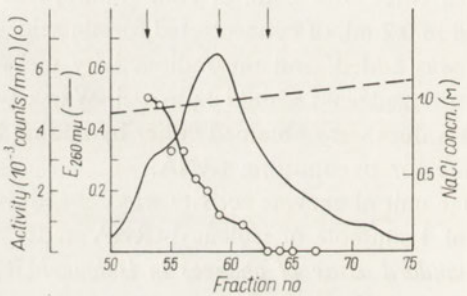

Fig. 2

Fig. 1. Inactivation of isoleucyl-tRNA synthetase as a function of the time of heating at 45°, (△) in the absence of tRNA, and in the presence of: (■), crude lupin tRNA preparation (2.5 mg./ml.); (□), 10 mM-ATP; (●), 10 mM-ATP and 60 μM-isoleucine; (○), 60 μM-isoleucine. For activity determination, to the sample heated without tRNA, 0.2 mg. tRNA was added. Other details are given in Methods.

Fig. 2. Fractionation of crude lupin tRNA on DEAE-cellulose column at 72°. tRNA, 17 mg., was applied to a column (1 × 18 cm.) provided with a water jacket and equilibrated with 20 mM-sodium-acetate buffer, pH 6.0, containing 0.5 M-NaCl and 1 mM-EDTA. The elution was carried out with a linear NaCl concentration gradient, fractions of 1.1 ml. being collected, and (—), the amount of RNA was measured at 260 mμ. tRNA present in the eluted fractions was precipitated with 2 vol. of cold ethanol, centrifuged and dried at room temperature. Each sample was dissolved in 100 μl. of water and (○), acceptor activity in 20 μl. portions was determined. Arrows indicate the fractions used for the experiment presented in Fig. 3.

Table 1

Effect of tRNA *on the stabilization of isoleucyl-*tRNA *synthetase against thermal inactivation*

The complete system for heat inactivation contained in 80 µl.: 10 µmoles of tris-HCl buffer, pH 7.5; 1 µmole $MgCl_2$, 1 µmole KCl, 0.2 mg. of crude lupin tRNA preparation, 1 µmole of 2-mercaptoethanol and 0.1 mg. of enzyme protein. After heating for the time indicated, the activity was measured as described in Methods. For activity determination, to the samples heated without tRNA, 0.2 mg. tRNA was added.

Composition of the heated sample	Time of heating at 50° (min.)					
	0	1	3	5	7	9
	Remaining activity (% of initial activity)					
Complete system	100	88	74	61	50	43
tRNA omitted	100	8	0	0	—	—
tRNA omitted, rRNA added	100	4	0	0	—	—
tRNA omitted, isoleucine and ATP added	100	12	0	0	—	—

0% of $tRNA^{Ile}$, were used for the experiment. The results presented in Fig. 3 show that the protection of the enzyme against heat denaturation was proportional to the content of $tRNA^{Ile}$. The fraction containing no $tRNA^{Ile}$ did not stabilize the enzyme.

These results were confirmed by using a highly purified (83%) $tRNA^{Ile}$ preparation (Fig. 4). When lysine-specific tRNA preparation purified to 75% and containing 2% of $tRNA^{Ile}$ was used, some protection was observed amounting to 4 - 5% of the action of $tRNA^{Ile}$.

The results suggest that a complex is formed between the synthetase and its specific tRNA, which stabilizes the protein component against thermal denaturation.

Specificity in complex formation. Synthetase - tRNA complexes were also formed with heterologous tRNA's. Figure 5 shows the effect of lupin, yeast and *E. coli* tRNA's, respectively, on the stabilization of isoleucine-activating enzyme. Apart from the fact that individual preparations might contain different amounts of isoleucine-specific tRNA, it seemed to be rather clear that complexes of enzyme with yeast and bacterial tRNA were considerably less stable than that containing homologous tRNA[2]. The half-inactivation times of the enzyme in the presence of lupin, yeast and *E. coli* tRNA preparations amounted to 7, 3.5 and 1.75 min., respectively.

[2] The determination of isoleucine acceptor activities of crude tRNA preparations using lupin enzyme showed considerably higher values for yeast and *E. coli* tRNA's than for lupin tRNA; which, however, is not equivalent with the higher content of $tRNA^{Ile}$ in these preparations.

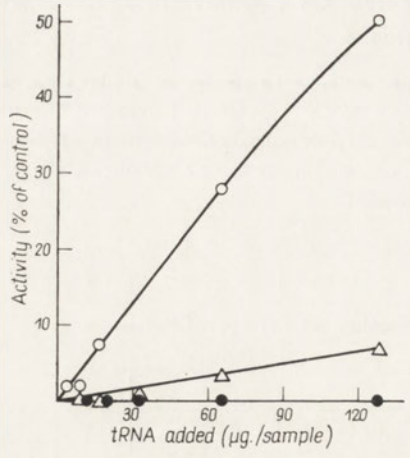

Fig. 3

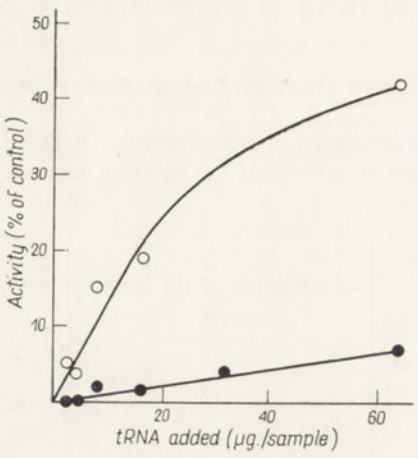

Fig. 4

Fig. 3. Effect of concentration of three lupin tRNA fractions (see Fig. 2) on the stabilization of isoleucyl-tRNA synthetase. To the standard inactivation mixture containing 0.2 mg. of the synthetase, indicated amounts of the following tRNA fractions were added: (○), 53rd, containing about 14% of $tRNA^{Ile}$; (△), 59th, 2% of $tRNA^{Ile}$; (●), 63rd, containing no $tRNA^{Ile}$. The mixture was heated for 5 min. at 50°, and the remaining activity was determined as described in Methods, except that 50 μg. of the purified preparation of $tRNA^{Ile}$ (20%) was added.

Fig. 4. Stabilization of isoleucyl-tRNA synthetase by purified preparations of (○), $tRNA^{Ile}$ and (●), $tRNA^{Lys}$. The experiment was carried out as described for Fig. 3.

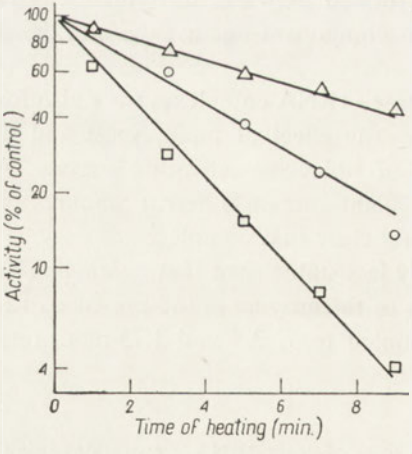

Fig. 5. Stabilization of isoleucyl-tRNA synthetase by homologous and heterologous tRNA's. The mixture described in Methods containing 0.2 mg. of crude tRNA preparation from (△), lupin; (○), yeast or (□), *E. coli* was heated at 50° for the time indicated. Then the remaining activity was determined. For details see Methods.

Effect of divalent and monovalent cations on the stabilization of isoleucyl-tRNA *synthetase* - tRNA *complex*. The stabilization of the enzyme by tRNA was dependent on the presence of Mg^{2+} ion (Table 2). Low concentrations of Mg^{2+} in the range up to 0.02 mM, were insufficient to co-operate with tRNA in enzyme protection. Among the concentrations applied, 10 mM-Mg^{2+} gave the best result. Differences in control values for enzyme activities shown in the Table were due to different Mg^{2+} concentrations being present. Magnesium ion could be substituted by Ca^{2+} or Sr^{2+} ions but not by ethylenediamine (Table 3).

Calcium and strontium ions, in contrast to their protective effect against thermal inactivation, could not replace magnesium ion in the tRNA aminoacylation reaction (Table 4), and their presence had even some inhibitory effect.

The effect of monovalent cations on isoleucyl-tRNA synthetase heat inactivation at 45° and on tRNA aminoacylation (Figs. 6 and 7), under the conditions used was very small, if any.

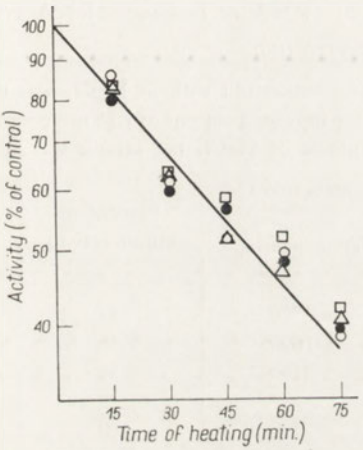

Fig. 6

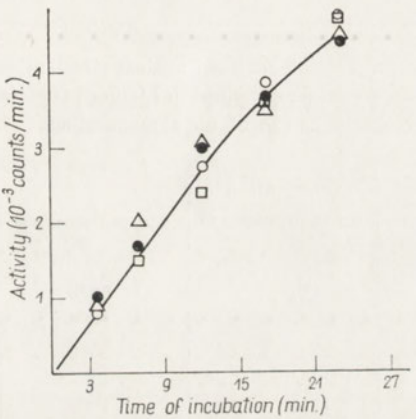

Fig. 7

Fig. 6. Effect of monovalent cations on the inactivation of isoleucyl-tRNA synthetase at 45° in the presence of tRNA. To the standard inactivation mixture but not containing KCl, one of the given salts was introduced at a concentration of 40 mM: (●), tris-HCl, pH 7.5; (□), NaCl; (○), KCl; (△), NH_4Cl. Determination of the remaining enzyme activity was carried out as described in Methods. For this experiment the enzyme preparation was dialysed against 20 mM-tris-HCl buffer, pH 7.3, containing 1 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol, and the crude preparation of tRNA was dialysed against 5 mM-tris-HCl buffer, pH 7.3, containing 1 mM-$MgCl_2$.

Fig. 7. Effect of monovalent cations on aminoacylation of lupin tRNA by the homologous isoleucyl-tRNA synthetase. To the standard reaction mixture but not containing KCl, one of the given salts was introduced to 40 mM concentration: (●), tris-HCl, pH 7.5; (△), NaCl; (○), KCl; (□), NH_4Cl. Enzyme and tRNA preparations were prepared as described for Fig. 6. The assay procedure was as described in Methods.

Table 2

Effect of $MgCl_2$ *concentration on the stabilization by* tRNA *of isoleucyl-*tRNA *synthetase against heat inactivation*

The inactivation mixture containing crude lupin tRNA was as described in Methods, except that varying amounts of $MgCl_2$ were present. The mixture was heated for 5 min. at 50°. For determination of the remaining enzyme activity, 0.8 µmole of $MgCl_2$ was additionally introduced to each sample.

$MgCl_2$ concn. in the heated mixture (mM)	Enzyme activity (counts/min.)		Percent of initial activity
	without heating	after heating	
0.01	6700	0	0
0.02	6600	0	0
1.2	7170	1590	22
10	5360	3410	64
62.5	3320	840	25

Table 3

Effect of divalent cations on the stabilization by tRNA *of isoleucyl-*tRNA *synthetase against heat inactivation*

Enzyme preparation dialysed against standard buffer solution but without $MgCl_2$ was used. The inactivation mixture described in Methods contained the indicated cations in 1.25 mM concentration. For determination of the remaining activity, 1 µmole of $MgCl_2$ per sample was added.

Inactivation in the presence of	Enzyme activity (counts/min.)		Percent of initial activity
	without heating	after heating	
$MgCl_2$	6550	3720	57
$CaCl_2$	1690	1000	59
$SrCl_2$	3680	2180	59
Ethylenediamine ·2HCl	1900	0	0

Table 4

*Effect of divalent cations on isoleucyl-*tRNA *synthetase activity*

Enzyme preparation dialysed against standard buffer solution but without $MgCl_2$ was used. The composition of the reaction mixture was as described in Methods, except that different cations were applied in place of $MgCl_2$.

Addition (0.8 mM)	Enzyme activity (counts/min.)
None	0
$MgCl_2$	5800
$CaCl_2$	310
$SrCl_2$	0
Ethylenediamine·2HCl	0

DISCUSSION

Recently several relatively stable complexes of aminoacyl-tRNA synthetases with their specific tRNA's were isolated by different methods such as gel filtration (Lagerkvist *et al.*, 1966), electrophoresis (Okamoto & Kawade, 1967; Seifert *et al.*, 1968) and adsorption on nitrocellulose filters (Yarus & Berg, 1967). The stabilization of the isoleucyl-tRNA synthetase against heat denaturation by its specific tRNA, demonstrated in this report, is a further, although indirect, proof of the formation of complexes between these components. The tRNA preparation deprived of the isoleucine-specific component appeared to be inactive in this respect, as was the ribosomal fraction of RNA. The ability of crude tRNA to protect an individual synthetase against heat denaturation is not a common property, like the ability to form enzyme - tRNA complexes. Thus lupin leucyl- and lysyl-tRNA synthetases were not stabilized by lupin tRNA.

The results concerning the dependence of isoleucyl-tRNA synthetase stability on tRNA concentration (Figs. 3 and 4), and the time-course of inactivation in the presence of tRNA (Fig. 1), are consistent with the model of a simple equilibrium: $E + tRNA \rightleftharpoons E\text{-}tRNA$, in which free enzyme would undergo rapid heat denaturation. The same model was accepted for the thermal stabilization of proline- and valine-activating enzymes by their amino acid substrates (Chuang *et al.*, 1967).

The most interesting observations made in our experiments concern the effect of divalent cations on the stability of isoleucyl-tRNA synthetase against heat inactivation. So far, the effect of these cations has been observed mainly on amino acid activation, transaminoacylation of tRNA, or the overall reaction. The activation process is dependent on the presence of magnesium ion, although magnesium can be sometimes replaced by manganese or calcium ions (Allende *et al.*, 1965; Svensson, 1967). Data relevant to the requirement of magnesium for the transfer reaction are contradictory. Norris & Berg (1964) and Lagerkvist *et al.* (1966), investigating *E. coli* isoleucyl- and valyl-tRNA synthetases, respectively, have shown that the reaction proceeds without magnesium. On the other hand, Allende *et al.* (1965) have reported that magnesium is required for rat liver threonyl-tRNA synthetase activity. Similar results were obtained by Hirsch (1968) with *E. coli* threonyl-tRNA synthetase.

In the present work it has been found that divalent cations are required even before the amino acid activation and tRNA aminoacylation reactions, namely for formation of the complex between isoleucyl-tRNA synthetase and $tRNA^{Ile}$. This complex would protect the enzyme against rapid heat denaturation. Since, besides magnesium, the strontium, which is inactive in the enzyme reactions, also fulfills the metal requirement, one can assume that the role of divalent cations consists mainly in the maintaining of the biologically active conformation of tRNA. On the other hand, the decrease of tRNA aminoacylation in the presence of strontium and calcium can be explained by the displacement of Mg^{2+} by Sr^{2+} or Ca^{2+} (Table 3) in this partially Mg^{2+}-dependent reaction. It should be emphasized that

studies of Fresco, Adams, Ascione, Henley & Lindahl (1966) on a thermostable aminoacyl-tRNA synthetase have shown that a unique tertiary conformation of tRNA is required for its accurate participation in the reaction.

Under conditions used in our work, no effect of monovalent cations was observed either on the inactivation or aminoacylation processes, although stimulation or inhibition by monovalent cations of the ATP-PP exchange reaction (Schweet, Holley & Allen, 1957; Schweet & Allen, 1958; Allen, Glassman & Schweet, 1960), transfer reaction (Svensson, 1967; Kaziro, Takahashi & Inon, 1968), as well as aminoacyl-tRNA formation (Peterkofsky, Gee & Jesensky, 1966; Smith, 1969) are well documented.

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STABILIZACJA IZOLEUCYLO-tRNA SYNTETAZY IZOLOWANEJ Z NASION ŁUBINU ŻÓŁTEGO PRZEZ TRANSFEROWY RNA

Streszczenie

tRNA ochrania izoleucylo-tRNA syntetazę izolowaną z nasion łubinu żółtego, przed termiczną inaktywacją zachodzącą w 50°. Efekt stabilizujący jest uwarunkowany obecnością tRNA specyficznego dla izoleucyny; inne rodzaje tRNA nie wykazują tego działania. Heterologiczne tRNA (z drożdży i komórek *E. coli*) również stabilizują badaną syntetazę lecz w mniejszym stopniu. Dla stabilizacji enzymu nieodzowna jest obecność dwuwartościowych kationów, jak Mg^{2+}, Ca^{2+} lub — nieaktywnego w procesie aminoacylacji tRNA — Sr^{2+}. Wskazuje to na rolę dwuwartościowych kationów w utrzymaniu trzeciorzędowej struktury cząsteczki tRNA, koniecznej dla powstania kompleksu tRNA - enzym.

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INVESTIGATIONS ON THE STRUCTURES OF XANTHINE-URACIL AND XANTHINE-ADENINE COPOLYMERS AND THEIR COMPLEXES WITH HOMOPOLYNUCLEOTIDES

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1. Polynucleotide phosphorylase has been used to synthesize a series of copolymers of xanthine with uracil or adenine. 2. For each copolymer with an excess of one component, the structure approximates that of the homopolymer of that component. 3. Hydrolysis of the copolymers with pancreatic and T_1 RNases was employed to examine the structures of the non-hydrolysed fragments. 4. Mixing curves and temperature profiles were used to examine the complexes of the copolymers with poly-X, poly-U and poly-A. 5. For 6 out of 16 complexes, both copolymer components were found to base-pair with the complementary chain. 6. The influence of various factors, including base sequence, is discussed in relation to the various structures, as well as their biological implications.

The results presented here on secondary structures of copolymers formed by polynucleotide phosphorylase from mixtures with differing relative contents of XDP[1] and UDP, or XDP and ADP, are a continuation of investigations on the structure of poly-X itself (Fikus & Shugar, 1969) and the interactions of xanthosine incorporated in the polynucleotide together with other purine and pyrimidine bases. The series of XU and XA copolymers thus obtained is an interesting subject of investigation on how the self-structure of a homopolynucleotide alters with increasing content of a heterologous (different base) component, and also how the comple-

[1] The following abbreviations are used in this text: poly-X, poly-riboxanthylic acid; poly-U, poly-ribouridylic acid; poly-A, poly-riboadenylic acid; poly-rT, poly-ribothymidylic acid; poly-XU_n (or copolymer XU_n or XU_n), copolymer of xanthine and uracil; poly-XA_n (or copolymer XA_n or XA_n), copolymer of xanthine and adenine; the subscript n refers to the percentage of the heterologous base component in the copolymer (uracil or adenine, respectively); poly-(XA:U), 1:1 complex of poly-XA and poly-U, with similar connotations for other copolymer or polymer complexes; ε, coefficient of molar absorption; T_m, temperature corresponding to mid-point of temperature profile; ΔT, measure of breadth of temperature profile, defined as temperature range over which the hyperchromicity of a given polymer or complex increases from 25% to 75% of the maximum hyperchromicity.

xing ability of each copolymer component in interactions with homopolynucleotide depends on the conditions of the medium and the percentage of a given component in the copolymer. The results allow for deeper penetration into the structure of the homopolynucleotide, poly-X, and its complexes; there are also attempts to explain on a simple model system the mutagenic effect of guanosine deamination to xanthosine incorporated into natural nucleic acids.

MATERIALS AND METHODS

Copolymers XU and XA were obtained by polymerization of mixtures of XDP (Sigma Chem. Co., St. Louis, Mo., U.S.A.) and UDP (C. F. Boehringer & Soehne, Mannheim, West Germany) or XDP and ADP (Sigma Chem. Co.) by polynucleotide phosphorylase from *M. lysodeikticus* (a gift of Dr. H. Matthaei). Poly-X was obtained as previously described (Fikus & Shugar, 1969) and the polynucleotides isolated. Poly-U and poly-A were commercial preparations of Miles Chemical Co. (Elkhart, U.S.A.). The commercial preparations were not purified further.

Polymer concentrations, and the percentage proportion of bases incorporated into the polymers, were determined spectrally, after enzymic or chemical hydrolysis to monomers. Copolymers were hydrolysed either in 0.3 M-KOH (AX series), monophosphates of both components being obtained, or at 100°, 1 hour, in 1 N-HCl (UX series) to uridylic acid and xanthine. The products of hydrolysis were chromatographically separated, eluted and spectrophotometrically estimated.

Enzymic hydrolysis was carried out with pancreatic RNase (Worthington, Freehold, N. J., U.S.A.) and RNase T_1 (Calbiochem., Los Angeles, Calif., U.S.A.) directly in spectrophotometric cuvettes. A Radiometer type 4d meter with glass electrode was employed for pH measurements. Ultraviolet spectra and temperature profiles were run on a Hungarian spectrophotometer "Spektromom 202" according to the method already described (Fikus & Shugar, 1969).

Sedimentation constants were measured with an analytical ultracentrifuge Beckman "Spinco E".

0.05 M-Acetate buffer was used within a pH range of 3.9 to 5.8 and 0.025 M-phosphate buffer within the pH range 6.0 to 8.0. Ion concentration (Na^+) was adjusted by adding the appropriate amount of 4 M-NaCl to the buffer.

The method of continuous variations (Fresco & Alberts, 1960) was used to observe formation of complexes, the reciprocal relation of components of the complex and also the extent of participation of each copolymer base in the formation of a complex with a copolymer.

RESULTS AND DISCUSSION

The spectra of all copolymers were investigated at room temperature at pH 2, 7 and 12. Since, in the majority of instances, these spectra depend not only on pH, but also on the state of the copolymer secondary structure, they do not provide

Table 1

Characteristics of copolymers

Copolymer	Composition of substrate mixtures (%)		Proportion of bases in the isolated copolymer (%)		S_{20} 0.05 M-Na^+, pH 7.8
	XDP	UDP	XDP	UDP	
XU_{84}	30	70	16	84	
XU_{88}	39	61	12	88	
XU_{69}	60	40	31	69	
XU_{20}	95	5	80	20	
	XDP	ADP	XDP	ADP	
XA_{87}	27	73	13	87	5.1
XA_{65}	47	53	35	65	5.1
XA_{32}	57	43	68	32	
XA_{26}	81	19	74	26	

sufficient information about the effect of the respective components on the shape of copolymer spectrum. Because of the considerable differences in coefficients of molar absorption of adenosine and xanthosine, in the AX series, the characteristic xanthosine absorption bands are usually hidden by the adenosine spectrum. In the XU copolymer series, in extreme instances of high percentage contents of one of the components, the copolymer spectra resemble the spectra of the homopolymer of the predominant incorporated nucleotide.

Detailed quantitative data on copolymer characteristics are presented in Tables 1 - 7 and Figures 1 - 14. On the whole, it may be inferred that the copolymer having a considerable predominance of one of the components exhibits a structure analogous to that of the homopolymer structure of the predominant component. And so, e.g., complete alkaline and enzymic hydrolysis of poly-XU_{88}, XU_{84} and XU_{69} results in an increase in the absorption maximum of some 5% (as for poly-U), which also demonstrates the lack of secondary structure of the copolymers at a temperature of 25°. Therefore, in order to check this structure, the copolymers were dissolved at pH 7.0 in 5×10^{-5} M-spermine. Fig. 1 and Table 2 illustrate the influence of spermine (or spermidine) and Na^+ ions on the stability of the structure of XU copolymers. Fig. 1 illustrates also an interesting phenomenon. As might have been expected, poly-XU_{88} and XU_{84} are of weaker structure than the homopolymer, poly-U, under identical conditions. But poly-XU_{69}, both in spermine and 0.1 M-Na^+, is characterized by a considerably higher melting temperature than the homopolymer, poly-U, though the breadth of the profile increases with increasing xanthosine content in the chain. It seems, at the same time, that the interaction U:U has a decisive influence on this structure, since the increase of the melting temperature of

poly-XU_{69} from 19° to 38° after the addition of spermine (up to 5×10^{-5} M) to 0.1 M-Na^+ resembles the behaviour of the homopolymer, poly-U (Szer, 1966). After the addition of spermine, poly-X melts at a temperature only 6° higher than in 0.1 M-Na^+, whereas for complex poly-(X:U) this values is 15° higher (cf. with Fig. 1).

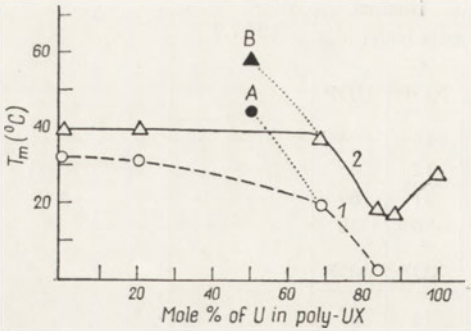

Fig. 1. Influence of Na^+ ions and spermine on stability of XU copolymers at pH 7.0: *1*, 0.1M-Na^+; *2*, 0.1 M-Na^+, 5×10^{-5} M-spermine. Point *A* denotes the T_m of poly-(X:U) at pH 7.0 in 0.1 M-Na^+. Point *B* denotes the T_m of poly-(X:U) at pH 7.0 in 0.1 M-Na^+, 5×10^{-5} M-spermine.

Thus the only explanation for the higher stability of poly-XU_{69} as compared to poly-U, at pH 7, would be to assume the existence of interactions reinforcing the structure of U:U type: either X:X interactions existing in "loops" formed by longer homologous sections of xanthosine, or X:U local interactions existing in some nodes, e.g. at the twist of the chain in the case of a "hairpin" structure. Similar loops would also explain why this entire structure melts less co-operatively than poly-U or poly-X alone.

Table 2

Characteristics of XU type copolymers with excess U in neutral phosphate buffered medium in the presence of Na^+ *ions and polyamines*

Polymer	Solvent	T_m (°C)	ΔT (°C)	Hyper-chromicity (%)	Wavelength at which hyperchromicity measured (mμ)
Poly-XU_{84}	pH 7.0; 0.1 M-Na^+	2.5	10	40	260
Poly-XU_{69}	pH 6.9; 0.1 M-Na^+	18	10	26	275
Poly-U*	pH 7.4; 4×10^{-5} M-spermidine	24	1.5	42	260
Poly-XU_{88}	pH 6.9; 0.05 M-Na^+; 5×10^{-5} M-spermidine	2	4.5	25	260
Poly-U*	pH 7.4; 4×10^{-5} M-spermine	28.5	1	42	260
Poly-XU_{88}	pH 6.9; 0.05 M-Na^+; 5×10^{-5} M-spermine	18	4	35	260
Poly-XU_{84}	pH 7.0; 0.1 M-Na^+; 5×10^{-5} M-spermine	19	14	40	260
Poly-XU_{69}	pH 6.9; 0.1 M-Na^+; 5×10^{-5} M-spermine	38	9	22	275

* Data from Szer (1966).

The secondary structure of poly-XA_{87} (and XA_{65}) was ascribed mainly to A:A interactions, as these polymers have a stepwise melting "neutral" structure (at pH > 6) and a co-operatively melting "acid" structure (at pH < 6), as for poly-A. Increasing ionic strength leads to a lowering of the stability of the "acid" structure, as in the case of poly-A, but in contrast to the behaviour of poly-X under these conditions (Figs. 2, 3, Table 3). Attention should be drawn to the fact that the transition temperatures for the "acid" structure of poly-XA_{87} are almost identical with those for poly-A (Table 3) whereas similarly perturbed $8BrA,A_{83}$ copolymer melts 13° below poly-A (Ikehara, Tazawa & Fukui, 1969).

Interactions of both bases in the copolymer, and also the influence of homologous sequences on the structure could also be examined by the application of specific enzymes. In the XU series, after the action of pancreatic RNase, the sequences $(Xp)_nUp$ remain unchanged; and after the action of RNase T_1 — $(Up)_nXp$ sequences. In the XA series RNase T_1 was applied to obtain $(Ap)_nXp$ sequences. The results on oligomers thus obtained confirm the assumption that both bases are randomly incorporated into the copolymer, despite the fact that polynucleotide phosphorylase differs in its affinity for them. And so, e.g., Fig. 4 shows that, after hydrolysis of poly-XU_{88} with RNase T_1, several per cent of sufficiently long sequences of oligo-U remained that they display very small proper structure. The failure to

Table 3

Comparison of "acid" forms of XA copolymers with excess A and the homopolymer poly-A

Solvent	Polymer	T_m (°C)	ΔT (°C)	Hyperchromicity (%)
pH 4.0; 0.05 M-Na^+	Poly-A*	>100	—	—
	Poly-XA_{87}	98	—	—
	Poly-XA_{65}	82	—	—
pH 4.4; 0.05 M-Na^+	Poly-A*	98	—	—
	Poly-XA_{87}	96	13	—
	Poly-XA_{65}	66	16	25
pH 4.8; 0.05 M-Na^+	Poly-A*	82	3	60
	Poly-XA_{87}	79	15	45
	Poly-XA_{65}	58	17	30
pH 5.2; 0.05 M-Na^+	Poly-A*	66	—	—
	Poly-XA_{87}	61	8	54
0.1 M-Na^+	Poly-A*	60	—	—
	Poly-XA_{87}	57	8	54
pH 5.8; 0.05 M-Na^+	Poly-A*	43	—	—
	Poly-XA_{87}	37	8	27

* Data from Massoulié (1965).

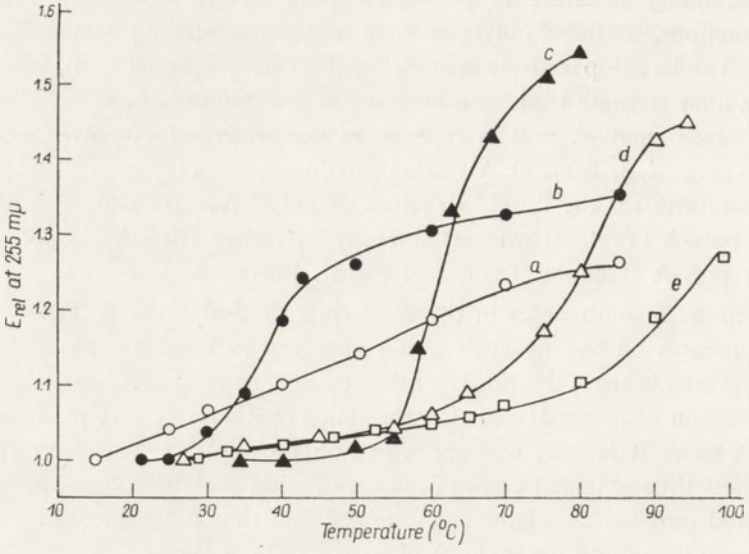

Fig. 2. Secondary structure of poly-XA_{87} in 0.05 M-Na^+ in (*a*) 0.025 M-phosphate buffer; (*b* - *e*) in 0.05 M-acetate buffer; (*a*), pH 7.8; (*b*), pH 5.8; (*c*), pH 5.2; (*d*), pH 4.8; (*e*), pH 4.4. Note: In this and following figures, E_{rel} is the ratio of the absorption at a given temperature to the absorption of the fully structured form, both measured at the same wavelength.

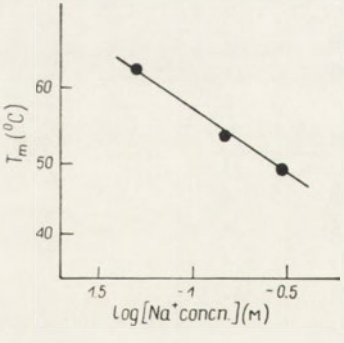

Fig. 3

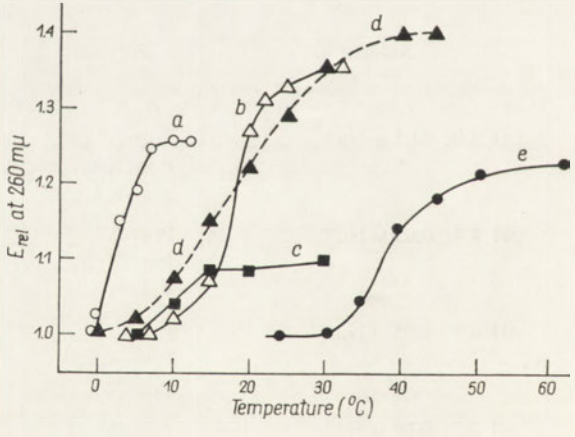

Fig. 4

Fig. 3. Dependence of T_m of poly-XA_{87} on Na^+ concentration in acetate buffer, pH 5.2.

Fig. 4. Secondary structure in 0.025 M-phosphate buffer, pH 6.9, and 0.05 M-Na^+ of (*a*, *b*, *c*) poly-XU_{88}, (*d*) poly-XU_{84}, and (*e*) poly-XU_{69}: (*a*), 5×10^{-5} M-spermidine; (*b*, *d*, *e*), 5×10^{-5} M-spermine; (*c*), as for (*b*) following digestion with RNase T_1.

obtain such a structure after the hydrolysis of poly-XU_{69} with RNase T_1 is consistent with the assumption regarding the random distribution of X and U residues along the chain. Similarly, the action of pancreatic RNase on poly-XU_{20} results in a hyperchromic effect which, at pH 7.8 at 5°, is equal to 15% at 275 mμ and 4% at 250 mμ (Total hyperchromicity of the copolymer is respectively 85% and 27%). The partially hydrolysed copolymer thus retained its proper structure typical for low-molecular poly-X (Fig. 5). But, when acting on the copolymer first with ribonuclease T_1, the maximum hyperchromic effect is obtained.

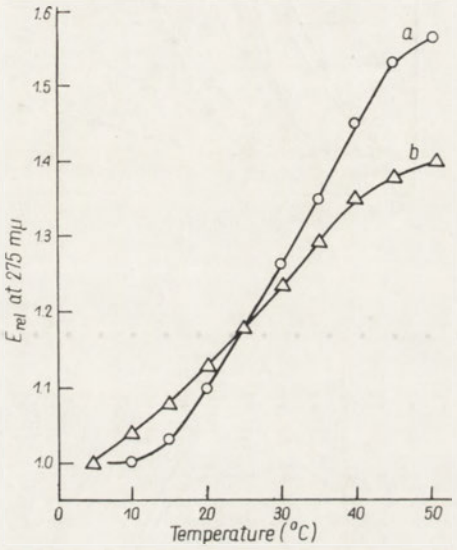

Fig. 5. Secondary structure of poly-XU_{20} in 0.025 M-phosphate buffer, pH 7.8, and 0.1 M-Na^+: (*a*), Control; (*b*), following digestion with pancreatic RNase.

Ribonuclease T_1 acts much slower on copolymer XA_{87} than on poly-X, and even after long incubation, its sedimentation constant and complexing ability with poly-U do not undergo any changes. But a visible effect after the action of RNase T_1 on poly-XA_{65} is observed *int. al.* due to considerable lowering of the co-operativity of melting of poly-(XA:U) complex.

Some additional information on the self-structure of the homopolynucleotide, poly-X, was obtained by investigating the copolymers XU_{20} and XA_{26}. Figure 6 exhibits the melting profiles of poly XU_{20}. Attention should be drawn to the shape of the profile at pH 7.0 (curve *b*): at first, as the temperature increases, the transition is fairly sharp; above the T_m it is broadened, and resembles the behaviour of some previously obtained preparations of poly-X which, on closer examination, proved to be of short chain length and polydisperse. The character of the profiles, measured at 250 mμ and 275 mμ, is similar to those of poly-X. Greater changes take place at 275 mμ. The profiles of poly-XU_{20}, especially in acid medium, confirm the previous proposal (Fikus & Shugar, 1969) that the reduction of ionization of the xanthosine rings (p*K* 5.5) reinforces the structure of poly-X sufficiently so that melting in buffer solution becomes gradually impossible. The U residues

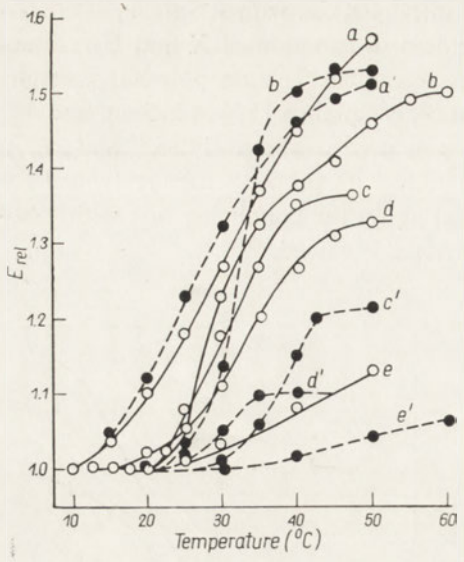

Fig. 6. Secondary structure of poly-XU_{20}. For comparison purposes, the dashed curves with primed letters refer to profiles of poly-X under same conditions: (*a*), 0.025 M-phosphate buffer, pH 7.8, and 0.1 M-Na^+; measured at 275 mμ. (*b*), 0.025 M-phosphate buffer, pH 7.0, and 0.1 M-Na^+; measured at 275 mμ. (*c*), 0.05 M-acetate buffer, pH 5.6, and 0.05 M-Na^+; measured at 260 mμ. (*d*), 0.05 M-acetate buffer, pH 5.2, and 0.05 M-Na^+; measured at 260 mμ. (*e*), 0.05 M-acetate buffer, pH 4.6, and 0.05 M-Na^+; measured at 260 mμ.

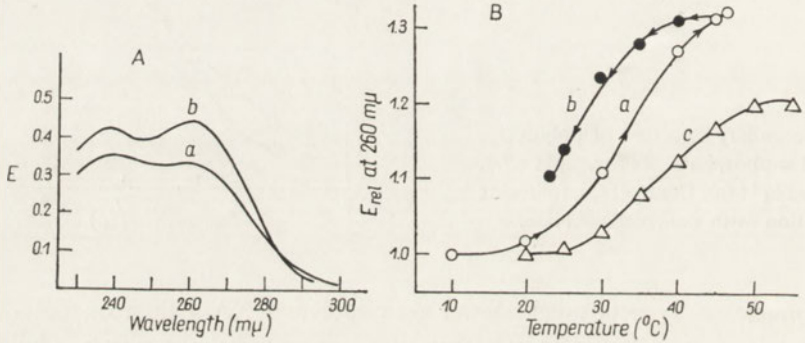

Fig. 7. Secondary structure of poly-XU_{20} in 0.05 M-acetate buffer, pH 5.2. *A*: (*a*), Spectrum of copolymer at 10° in presence of 0.05 - 0.15 M-Na^+; (*b*) copolymer spectrum at 46° in 0.05 M-Na^+. *B*: (*a*), Temperature profile in 0.05 M-Na^+; (*b*), cooling profile in 0.05 M-Na^+; (*c*), temperature profile in 0.15 M-Na^+.

present in the copolymer XU_{20} separate the longer structural sections X:X, and therefore weaken the strong "acid" structure, enabling its observation at pH 5.6 and 5.2 to a greater extent than for poly-X alone. The melting profile of poly-XU_{20}, at pH 5.6 (curve *c*), i.e. at pH = p*K* of xanthosine, is even almost identical with the profile at pH 7.0 and only acidification to pH 4.6 results in the disappearance of the co-operative transition during melting of the structure. It is worth noting that T_m values of poly-XU_{20} and X are almost identical. In the acid range only hyperchromicity underwent a change (Table 4). [Measurements of temperature changes were carried out at both maximum wavelengths for the copolymer, the figure (for clarity) presenting only curves at 260 mμ].

One additional fact is worthy of note, and is illustrated in Fig. 7A. Whereas the ordered structures of poly-XU_{20}, pH ~ 5, have a higher absorption at 240 mμ than at 260 mμ, during the heating this ratio changes in favour of the 260 mμ peak. Thus for the first time the high 260 mμ peak has been proved for poly-X at pH < 6, in a coil form, also present at these pH values in the xanthosine spectrum ($\varepsilon_{260} > > \varepsilon_{240}$). Heating of poly-$XU_{20}$ at pH 5.2, in the presence of 0.05 M-Na^+, above 60°, leads to irreversible changes in the spectrum (increase in ε_{260} in relation to ε_{240} also at a low temperature) and profile (lowering of T_m, broadening of the profile), which is probably due to shortening of the copolymer chain, weakening of "acid" structure and the appearance of spectral changes typical for monomers, and probably oligomers.

Profiles run at pH 5.6 - 4.6 up to 50°, are reversible, but the cooling profile is not identical with the heating profile (Fig. 7B). The reformation of structure can be quickened by changing the cation concentration from 0.05 M to 0.15 M, but in the new conditions, apart from T_m increasing 4°, the same thing happens which has been observed in the instance of poly-X, i.e. lowering of melting hyperchromicity (smaller per cent of structure is melted) and a decrease in co-operative character of the transition (Fig. 7B).

Table 4

Comparative data for self-structure of poly-XU_{20} and poly-XA_{26} with respect to poly X

Solvent	Polymer	T_m (°C)	ΔT (°C)	Hyper-chro-micity (%)	Wavelength at which hyperchromicity measured (mμ)
Phosphate buffer, pH 7.8; 0.1 M-Na^+	Poly-X	28	13	52	275
	Poly-XU_{20}	31	17	57	
	Poly-XA_{26}	26	15	45	
Phosphate buffer, pH 7.0; 0.1 M-Na^+	Poly-X	33	4	50	275
	Poly-XU_{20}	31	13	50	
	Poly-XA_{26}	33	8	27	
Acetate buffer, pH 5.6; 0.05 M-Na^+	Poly-X	36	6.5	22	260
	Poly-XU_{20}	31	10	26	
0.1 M-Na^+	Poly-X	38	6	21	
	Poly-XA_{26}	41	19	29	
0.15 M-Na^+	Poly-X	40	6.5	20	
	Poly-XU_{20}	35.5	12	36	
Acetate buffer, pH 5.2; 0.05 M-Na^+	Poly-X	30	2.5	10	260
	Poly-XU_{20}	33	10	32	
Acetate buffer, pH 4.6; 0.1 M-Na^+	Poly-X	does not melt			
	Poly-XA_{26}	70	17	41	260

In comparable conditions poly-XA_{26} behaves similarly to poly-X (Table 4). And also in this instance selective hydrolysis of the polymer with ribonuclease T_1 leads to a hyperchromic effect equal to that following complete hydrolysis of the polymer to mononucleotides (40% at 255 mμ and 45% at 275 mμ). At pH 4.6 poly-XA_{26}, like poly-XU_{20}, displays a secondary structure which can be melted. Thus it seems that the self-structure of copolymer XA_{26} is mainly due to X:X interactions, perturbed by short A sections. This is the reason for the co-operative transitions being lower than for the homopolymer, especially at acid pH. This is also confirmed by the fact that an increase in ionic strength, while melting the copolymer, increases its melting temperature, both at pH 7.8 and 4.6.

It is also worth noting that, both for the copolymers, and their complexes with homopolymers, to be discussed below, the cooling profiles usually differed from the first heating profile (cf. e.g. Fig. 7B), and the second melting profile was usually different from the first one. This phenomenon may be ascribed to the thermolability of phosphodiester bonds as in the case of the homopolynucleotides poly-X and poly-A (Barszcz & Shugar, 1964; Fikus & Shugar, 1969) (at neutral pH above 60° and 70°, respectively). It is also possible that the copolymer, consisting of two randomly distributed residues, renaturates with greater difficulty than a homopolymer, and it would thus be impossible (especially in the case of complexes with homopolymers) to reproduce, after cooling and before heating again, a structure identical with the initial one. This may be reflected in the changes of the spectra of the complexes before heating, and after heating and cooling. It could be a simple model of renaturation of natural nucleic acids, which are polydisperse from the standpoint of primary structure.

Therefore all experiments were made on samples melted only once. While measuring sedimentation constants, in the entire series of copolymers, some degradation was noted during storage of the preparations, which was also observed in the case of poly-U and poly-rT, but was attributed to the action of endonuclease, not isolated from preparations, after their synthesis during deproteinization. Because of the already mentioned thermolability of sequences -XpX-, -ApA- and probably -UpXpU- and -ApXpA- the stability of polynucleotide preparation could not be improved by attempted inactivation of the hypothetical enzyme at 100° (as is usually done with poly-U or poly-rT preparations).

Complexes of copolymers XU and XA with homopolynucleotides

Complexes of copolymers of both XU and XA series with homopolynucleotides appear to be promising, because of the high potential possibility of formation of base-pairs by hydrogen bonding of xanthine with other bases.

Thus, from a theoretical point of view, copolymers XU and XA should form complexes with poly-U and analogues, and with poly-A and poly-X. Whether both copolymer components will complex with homopolymer depends on steric considerations and also thermodynamic parameters of the system.

Table 5

Characteristics of complexes obtained, with respect to proportion of bases in complexes and formation of loops by base residues

Polynucleotide mixture	Complexing conditions	In the complex take part:	The loops were formed by:
$XU_{69}+A$	pH 7.0, 0.1 M-Na^+	(1A:1U)	X residues
$XU_{20}+A$	pH 7.0, 0.2 M-Na^+	(2XU:1A)	—
$XU_{20}+U$	pH 7.8, 0.1 M-Na^+	(1X:1U)	U residues
$XA_{87}+U$	pH 7.8, 0.05 M-Na^+	(1XA:1U)	—
	pH 7.8, 0.3 M-Na^+	(1XA:2U)	—
$XA_{87}+X$	pH 7.0, 0.1 M-Na^+	(1XA:2X)	—
$XA_{65}+U$	pH 7.8, 0.05 M-Na^+	(1A:1U)	X residues
$XA_{65}+X$	pH 7.0, 0.1 M-Na^+	(1A:2X)	X residues
$XA_{26}+U$	pH 7.8, 0.1 M-Na^+	(1X:1U)	A residues
$XA_{26}+A$	pH 7.0, 0.2 M-Na^+	(2X:1A)	A residues
XU_{88} and $XU_{84}+A$	pH 7.0, 0.1 M-Na^+	(1A:1U)	X residues
		(1A:2U)	X residues
	pH 7.0, 1 M-Na^+	(1A:1XU)	?
		(1A:2U)	X residues
	5×10^{-5} M-spermine	(1A:1XU)	?
		(1A:2U)	X residues

Table 5 presents a comparison of complexes obtained, complexing conditions and conclusions *re* the structure of the complexes obtained. It shows that if a copolymer contains at least 65% of one component, this major component can interact with the complementary base of the added homopolymer. It can also be seen that in the majority of instances, and as is also reported in the literature (Ichikawa, Fujita, Matsuo & Tsuboi, 1967; Tsuboi, Matsuo & Nakanishi, 1968), the second component does not react with the homopolymer, thus probably forming extra-structural loops. These conclusions are based on mixing curves of complexing components, and also follow from comparisons of transition temperatures and the profile breadths of the complexes with appropriate homopolymer complexes (cf. Tables 6 and 7 and Figs. 8 - 10, 12 - 14). But in no less than 6 instances, both melting profiles and mixing curves suggest that the two copolymer components take part in the formation of secondary structure with the homopolymer. Therefore these instances call for special attention.

Complex ($2XU_{20}$:A) is similar to the homopolymer complex (Fikus & Shugar, 1969) (Fig. 8) i. e. formed in two stages. It is worth recalling that there are theoretical possibilities of interactions 2U:1A and 2X:1A, but, without constructing a space-filling model, it cannot be established whether in the complex ($2XU_{20}$:1A) *all* the accessible bases are hydrogen bonded. It can be assumed e.g. that in the initially formed complex (XU_{20}:A), both X and U binds *via* hydrogen bonds with A, but after the rearrangement (XU_{20}:A)+XU_{20} → ($2XU_{20}$:A) the second chain XU_{20}

Table 6

Comparison of interaction in 0.025 M-*phosphate buffer*, pH *7.8, of complementary pair X:U in various complexes as indicated*

Complex	M-Na^+	T_m (°C)	ΔT (°C)	Hyperchromicity at 275 mμ (%)
(X:U)	0.1	46	1.5	57
(XU_{20}:U)	0.1	41	8.5	57
(XU_{84}:X)	0.1	33	11.0	46
(X:U)	0.15	48	1.5	57
(XU_{20}:U)	0.15	44	4.5	57

Table 7

Transition temperatures of various complexes of poly-A with poly-U, poly-A with poly-XU_{88}, and poly-A with poly-XU_{84}, all in 0.025 M-*phosphate buffer*, pH *7.0*

Conditions for complex formation	Complex components	Type of transition			
		(A:U) → A+U	2 (A:U) → (A:2U)	(A:2U) → (A:U)+U	(A:2U) → A+2U
0.1 M-Na^+	A+U	54		51	
	A+XU_{84}	42		32	
	A+XU_{88}	45		30	
1 M-Na^+	A+U		45		85
	A+XU_{84}		50		58
0.1 M-Na^+; 10^{-3} M-spermine	A+U		39		83
5×10^{-5} M-spermine	A+XU_{84}		38		57
	A+XU_{88}		46		71

becomes extended, without forming loops, along the existing complex (XU_{20}:A) — and the hydrogen bonds are formed only between the X residues of the second copolymer chain and the A residues of the homopolymer.

Figure 8 exhibits the formation and melting processes of the complex poly-($2XU_{20}$:1A) in 0.2 M-Na^+, pH 7. A similar experiment was carried out in 0.05 M and 0.1 M-Na^+ — with a rather surprising result. As shown by melting profiles over the entire investigated range of cation concentration (0.2 - 0.05 M), only the complex of type ($2XU_{20}$:1A) is formed, but the cation concentration has hardly any influence on the stability of the complex. T_m values in 0.05 M, 0.1 M and 0.2 M-Na^+ are 84, 86 and 86°, respectively. T_m values of poly-(2X:1A), under the same conditions, were 84, 80 and 74°, respectively (Fikus & Shugar, 1969). These data provide additional

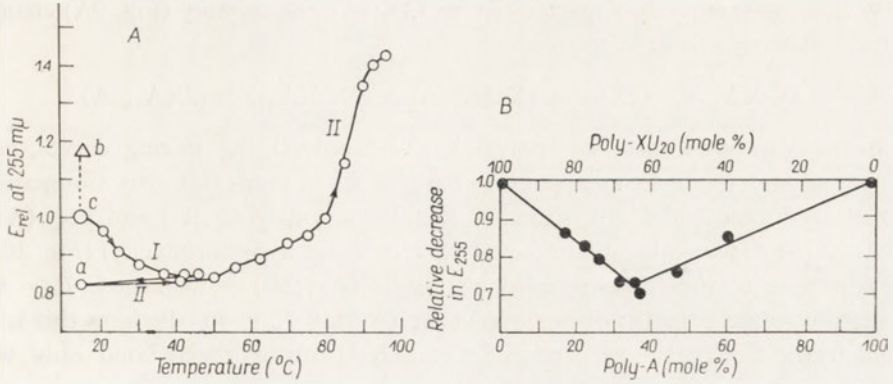

Fig. 8. Complex formation between poly-XU_{20} and poly-A in 0.025 M-phosphate buffer, pH 7.0, and 0.2 M-Na^+. *A*: Poly-XU_{20} mixed with poly-A in ratio 2:1 to give an absorption at 255 mμ denoted by point *c*. Point *b* denotes arithmetic sum of components. Solution heated to 47° (transition *I*), cooled to 15° (point *a*) and again heated to give profile *II*. *B*: Mixing curve of equimolar solutions of poly-XU_{20} and poly-A.

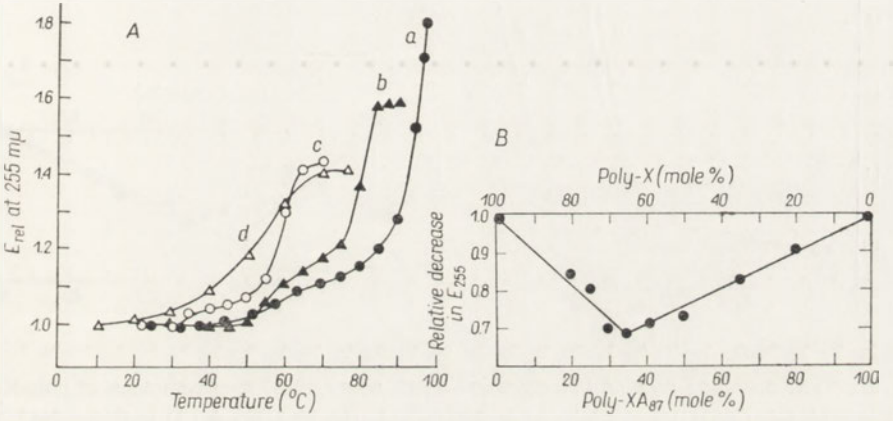

Fig. 9. *A*: Temperature profiles, in 0.025 M-phosphate buffer, pH 7, and 0.1 M-Na^+ of: (*a*), (2X:XA_{87}); (*b*), (2X:A); (*c*), (2X:XA_{65}); (*d*), (2XA_{26}:A) in 0.2 M-Na^+. *B*: Mixing curve of poly-X and poly-XA_{87} at pH 7.0 and 0.1 M-Na^+.

evidence that the U residues also participate in the formation of poly-(2XU_{20}:1A), as the influence of increasing concentrations of univalent cations on the A:U pair is stabilizing, and on the pair X:A — destabilizing.

Among the group of complexes of type (2X:1A) note in particular the complex (2X:XA_{87}) (Fig. 9A, curve *a*), which melts at considerably higher temperatures than the homopolymer complex (2X:1A) (Fig. 9A, curve *b*). At the same time the mixing curve points to the absence of loops in the polymer (Fig. 9B). These data suggest that, in this instance, the assumption concerning the spatial structure should be different than for the homopolymers, *int. al.* the interactions in one plane of three X residues would have to be taken into consideration.

Within the group of complexes of type (2X:1A), the stability (Fig. 9A) changes in the following order:

$$(2X:XA_{87}) > (2X:A) \approx (2XU_{20}:A) > (2X:XA_{65}) > (2XA_{26}:A).$$

In the case of complexes of poly-XA_{87} with poly-U, the mixing curves, both at low and high ionic strength, point to the absence of loops (Fig. 10). Comparison of melting profiles of double-stranded complexes poly-(XA_{87}:U) and poly-(A:U) points to their identical stability (equal T_m values and hyperchromicity) (Fig. 10A). The structure of the triple-stranded complex (XA_{87}:2U) is weakened, $T_m = 61°$, in relation to the complex of homopolymers (A:2U), $T_m = 70°$. Perhaps this is relevant to the fact that the second chain of poly-U can hydrogen bond only with

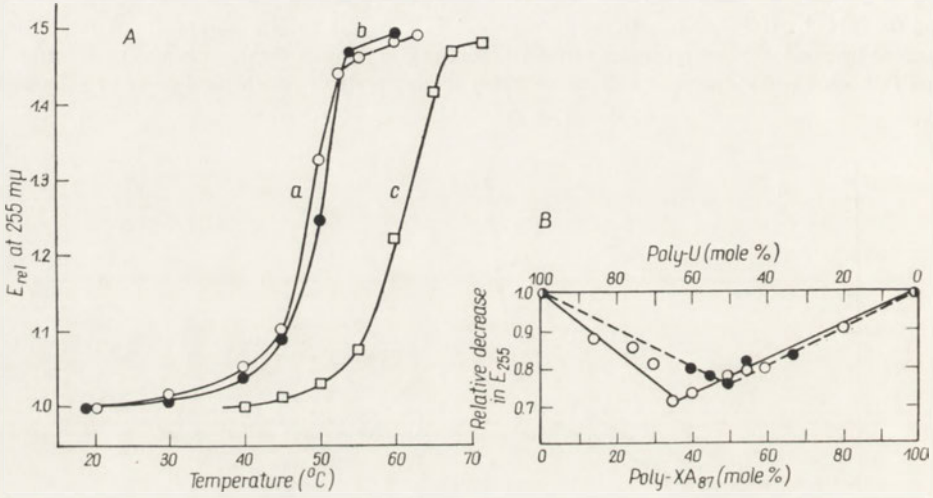

Fig. 10. *A*: Temperature profiles, in 0.025 M-phosphate buffer, pH 7.8, of complexes of poly-XA_{87} and poly-U: (*a*), (XA_{87}:U) → XA_{87} + U in 0.05 M-Na^+; (*b*), (A:U) → A + U in 0.05 M-Na^+; (*c*), (XA_{87}:2U) → XA_{87} + 2U in 0.3 M-Na^+. *B*: Mixing curves of equimolar solutions of poly-XA_{87} and poly-U at pH 7.8: (○), 0.05 M-Na^+; (●), 0.3 M-Na^+.

A, and not with the X of copolymers. Hence, taking into consideration the mixing curve, it is conceivable in this instance that the third chain is stretched along the double-stranded complex and bonded with it by U:A interactions. This recalls the structure of the complex (8BrGA_{77}:2U), suggested by Ikehara *et al.* (1969), in which after attaining equilibrium, 8Br-guanosine does not form loops outside the structure (the authors arrived at this conclusion by analysing mixing curves of the components), notwithstanding that the complex melts 5 - 8° lower than the complex of homopolymers (2U:1A).

In a group of complexes of (U:A) and (2U:A) type, when either XU_{84} or XU_{88} are the copolymers, the mixing curves are complex, and only the shape of profiles

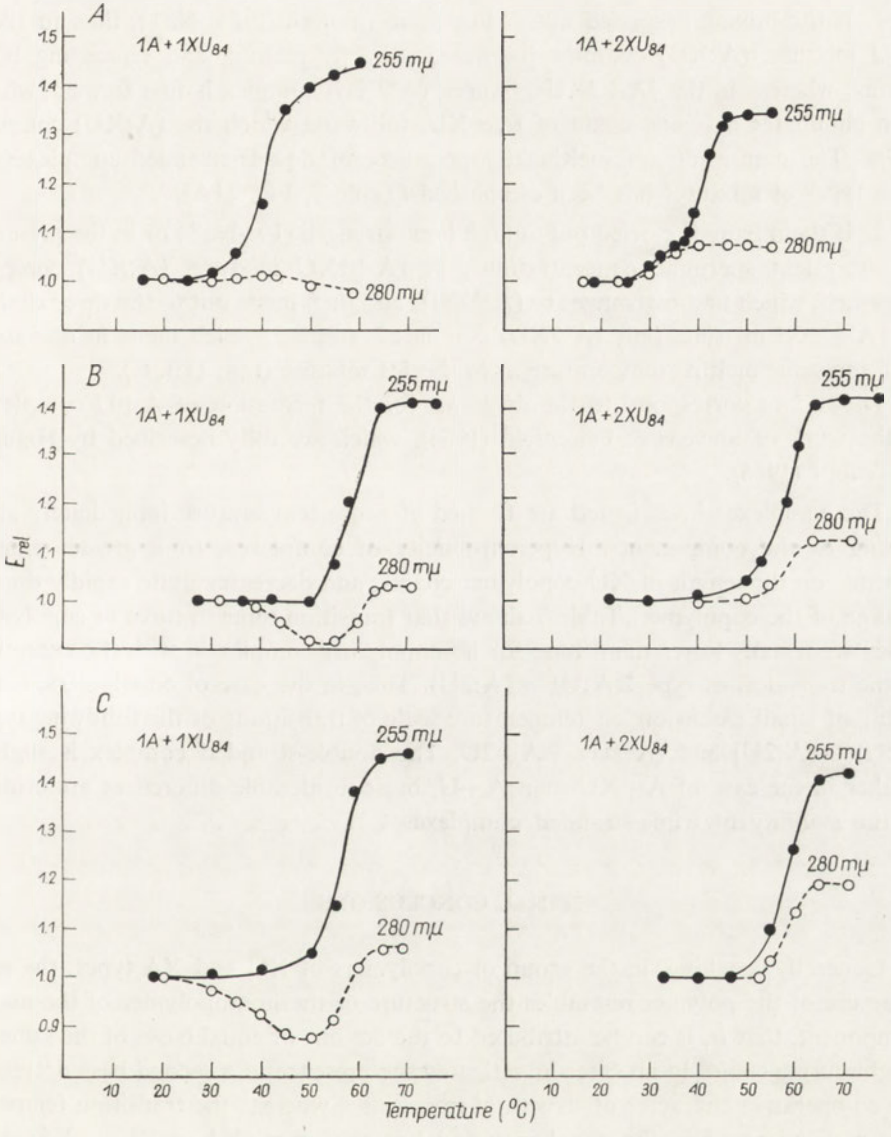

Fig. 11. Temperature profiles in 0.025 M-phosphate buffer, pH 7.0, of poly-(A:XU_{84}) at indicated percent ratios of components and wavelengths of measurement: *A*: 0.1 M-Na^+. *B*: 1.0 M-Na^+. *C*: 0.1 M-Na^+, 5×10^{-5} M-spermine.

and the T_m values may indicate, as denoted by question marks in Table 5, that there are no loops in the case of (A:XU) complex in 1M-Na^+ or 5×10^{-5} M-spermine (Fig. 11). From the experiments, it follows that:

1. If the mixing is carried out at low ionic strength (0.1 M-Na^+), then in 1A+1XU mixture, (A:XU) complex is formed, directly melting and separating both chains; whereas in the 1A+2XU mixtures, (A:2XU) complex is first formed, which then eliminates first one chain of free XU, following which the (A:XU) complex melts. The conformity of melting temperatures of double-stranded complexes in both types of mixtures has been established (Table 7, Fig. 11A).

2. If the mixing is carried out at high ionic strength (1 M-Na^+) or in the presence of equivalent spermine concentrations, in 1A+1XU mixture, (A:XU) complex is formed, which first rearranges to (A:2XU); and then melts out to the three chains. In 1A+2XU mixture, only (A:2XU) complex is formed, which melts in one stage with the same melting temperature as in the 1:1 mixture (Fig. 11B, C).

These data correspond to the diagrams for the formation of A+U complexes in the work of Stevens & Felsenfeld (1964), which are fully described by Higuchi & Tsuboi (1966).

The complexes investigated are formed at room temperature immediately after mixing of the components. Hyperchromicity of complexes, to a greater extent, depends on the length of XU copolymer chains, and decreases quite rapidly during storage of the copolymer. Table 7 shows that transition temperatures in copolymer series are usually lower than those for homopolymer complexes, with the exception of the transition of type 2(A:U) → (A:2U). This, in the case of copolymers, is the result of small extension on temperature scale of transitions of the following type: (A:U) → (A:2U) and (A:2U) → A+2U. The double-stranded complex is slightly weaker in the case of A+XU than A+U, but considerable differences are evident in the stability of triple-stranded complexes.

FINAL CONCLUSIONS

Generally speaking, in the group of copolymers of XU and XA types, the self-structure of the polymer resembles the structure of the homopolymer of the major component, that is, it can be attributed to the actions of equal bases of the same or neighbouring chain. In all cases investigated the presence of a second base decreases the co-operative character of structural transitions, whereas the transition temperature may be equal in the copolymer and homopolymer (XA_{87}, XU_{20}, XA_{26}) or even higher in the copolymer (XU_{69}). These facts suggest the possibility of stabilizing secondary structures by heterologous interactions, e.g. X:U or X:A. Thus it cannot be excluded that copolymer secondary structures differ from structures of corresponding homopolymers e.g. they are formed within the same chain instead of between two separate chains.

It has been shown that investigations on copolymer secondary structure may throw more light on homopolymer structure investigated under the same conditions

(e.g. poly-XU_{20} and poly-X at pH < 6). The present results also point to the possibility of complex formation between one copolymer component and its complementary homopolymer, under conditions where the copolymer contains at least 65% of this component. The structures of such complexes (at a considerable content of the second non-complexing component in the copolymer) melt less co-operatively, and the transition temperatures are markedly lower than the melting temperatures of homopolymer complexes. Mixing curves point to the formation of loops outside the structure by non-complexing bases. In the experiments the existence was also found of complexes which, it seems, involve the participation of both copolymer components. In these instances the structural transition is as co-operative as in the homopolymer complex, and the T_m values of the transitions equal, or even higher. None of these complexes can be described in terms of planar base models and stabilizing interactions by hydrogen bonding in one plane.

The comparison of complexes similarly perturbed, e.g. (XA_{26}:U) and (XU_{84}:X) is also interesting; judging by the course of melting curves, less perturbed structure is that of a copolymer composed of two purines than of a purine and pyrimidine (Fig. 12). Similarly, in double-stranded complexes of poly-(XU_{69}:A) and poly-(XA_{65}:U) (Figs. 13 and 14), more stable is the complex in which the copolymer is composed of two purines, although in both instances the X residues form loops outside the ordered structure.

Smaller perturbances of structure are observed in the ($2X:XA_{65}$) complex than in ($2XA_{26}$:A) (Fig. 9). In the second complex the various loops are probably formed

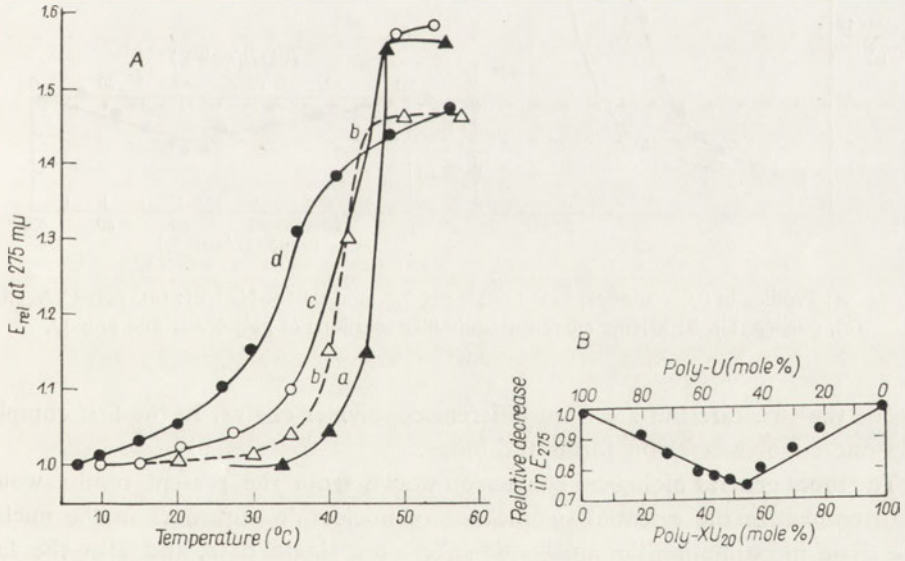

Fig. 12. *A*: Temperature profiles, in 0.025 M-phosphate buffer, pH 7.8, and 0.1 M-Na^+ of (X:U) type complexes: (*a*), poly-(X:U); (*b*), poly-(XA_{26}:U); (*c*), poly-(XU_{20}:U); (*d*), poly-(XU_{84}:X). *B*: Mixing curve of poly-U and poly-XU_{20}.

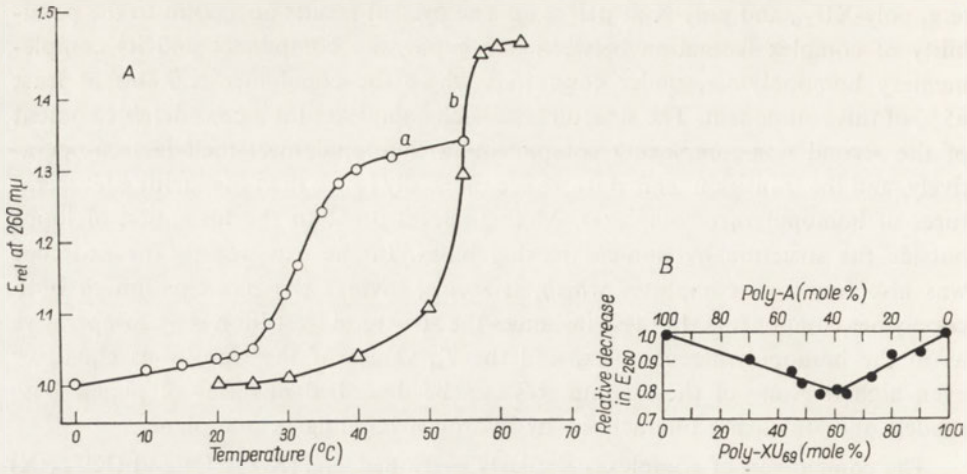

Fig. 13. Secondary structure of poly-(XU_{69}:A) in 0.025 M-phosphate buffer, pH 7.0 and 0.1 M-Na^+: *A*: (*a*), mixture of 60% XU_{69} and 40% A; (*b*), poly-(A:U). *B*: Mixing curve for poly-XU_{69} and poly-A.

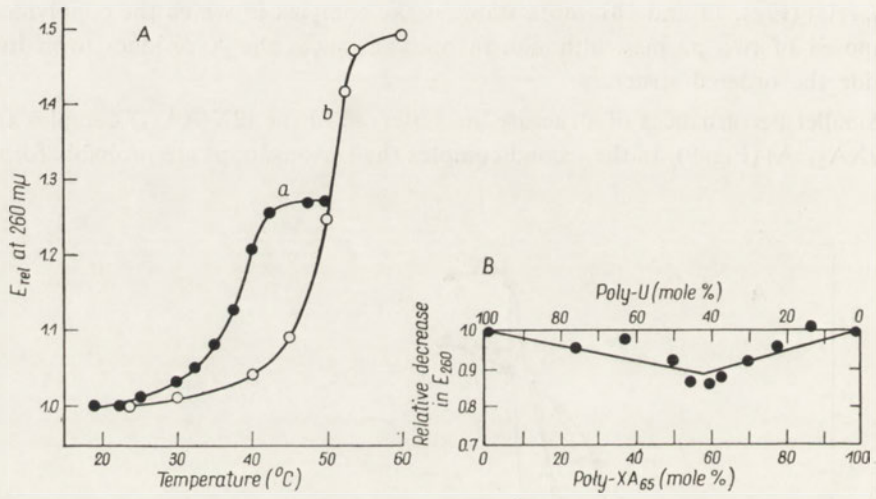

Fig. 14. *A*: Profiles in 0.025 M-phosphate buffer, pH 7.8, and 0.05 M-Na^+ of: (*a*), poly-(XA_{65}:U); (*b*), poly-(A:U). *B*: Mixing curve for equimolar solutions of poly-XA_{65} and poly-U.

outside the structure but are from different copolymer chains. In the first complex only one copolymer chain forms the loops.

The more general biological conclusion drawn from the present results would be to emphasize the essential significance of nucleotide sequences in the nucleic acid chain in estimating the mutagenic effect on a single base, and also the fact that the formation of xanthosine residues in the chain as a result of mutation does not necessarily lead to disruption of the structure in this region of the molecule by ejecting xanthine to an extrastructural loop.

However, all the conclusions from the present experiments must be formulated with reserve, since we must bear in mind that not much is known about the primary copolymer structures, apart from the *a priori* assumption that they are formed by random distribution of both residues, and the data on secondary structure are inferred indirectly from absorption spectroscopy. It is likely that these conclusions could be extended with the aid of ORD and CD measurements.

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BADANIE STRUKTUR KOPOLIMERÓW KSANTYNY Z URACYLEM I KSANTYNY Z ADENINĄ ORAZ ICH KOMPLEKSÓW Z HOMOPOLINUKLEOTYDAMI

Streszczenie

1. Za pomocą fosforylazy polinukleotydowej z *M. lysodeikticus* przeprowadzono syntezę szeregu kopolimerów ksantyny z uracylem lub adeniną.

2. Stwierdzono, iż w przypadku kopolimeru o przewadze jednego ze składników struktura własna kopolimeru przypomina strukturę odpowiedniego homopolinukleotydu.

3. Zbadano wpływ selektywnej hydrolizy kopolimerów specyficznymi enzymami (RNaza trzustkowa i T_1) na strukturę własną niestrawionych fragmentów kopolimeru.

4. Zbadano szereg kompleksów kopolimerów z potencjalnymi komplementarnymi homopolinukleotydami, poli-X, poli-U i poli-A. Oddziaływania obu składników kopolimeru z homopolimerami oceniano metodą zmian ciągłych i poprzez pomiary profili temperaturowych.

5. Wykazano, że w 6 na 16 zbadanych kompleksów istnieją podstawy do przypuszczeń, iż obie zasady kopolimeru oddziałują z zasadą homopolimeru.

6. Dyskutuje się wpływ środowiska oraz sekwencji kopolimeru na jego możliwości strukturotwórcze, a także ewentualne implikacje biologiczne stwierdzonych zjawisk.

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RECENZJE KSIĄŻEK

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS. Vol. X, part 3 (R. T. Holman, ed.). L. D. Bergelson: Diol Lipids. F. Snyder: The Biochemistry of Lipids Containing Ether Bonds. Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1969; str. 100; cena 42.—s, 5.00 $.

Omawiana książka zawiera kolejne dwie monografie w ramach dużej serii wydawanej od wielu lat a poświęconej postępom chemii i biochemii lipidów.

Pierwsza monografia dotyczy lipidów diolowych, to jest związków, w których komponentą alkoholową są dwuhydroksylowe pochodne etanu, propanu, butanu itp. Przez wiele lat, mimo stosowania subtelnych metod rozdzielczych, uchodziły one uwadze badaczy. Stanowią one bowiem niewielki odsetek lipidów naturalnych, a właściwościami są bardzo zbliżone do glicerydów. Od niespełna dziesięciu lat zaczęły pojawiać się doniesienia o obecności alkoholi dwuhydroksylowych w hydrolizatach naturalnych tłuszczów. Drogą pośrednią wydedukowano istnienie naturalnych lipidów dwuhydroksylowych analogów trójglicerydów, fosfolipidów, pochodnych eterów zarówno alkilowych, jak i alk-1-enylowych oraz innych lipidów. Niektóre z lipidów diolowych udało się wyizolować w czystej postaci, a ich budowę potwierdzić syntezą. Dużą część monografii autor poświęcił zagadnieniom chemicznym i metodycznym. Szeroko zostały omówione takie zagadnienia, jak izolowanie, różnego rodzaju rozdziały chromatograficzne, własności fizyczne a także sprawy związane z syntezą omawianych związków. Aspekty biochemiczne lipidów diolowych zmieściły się zaledwie na dwóch stronach. Nie sposób z tego jednakże zrobić autorowi zarzutu, należy jedynie uzmysłowić sobie, jak nikłe wiadomości posiadamy na temat przemiany tych związków. Piśmiennictwo obejmuje 95 pozycji.

Druga monografia dotyczy związków odkrytych już dawno, których najbardziej znanymi przedstawicielami są plazmalogeny. Plazmalogeny (alk-1-enyl glicerofosfatydy) były przedmiotem wielu wyczerpujących opracowań. Autor postanowił dokonać ogólnego przeglądu wszystkich lipidów zawierających wiązania eterowe. Dotyczy to alkilowych i alk-1-enylowych pochodnych monoacyloglicerofosfatydów, mono- i dwuacyloglicerydów a także lipidów diolowych. Rozdział pierwszy wprowadza ład w dość zagmatwanej nomenklaturze omawianych związków; następne zawierają dane o rozmieszczeniu lipidów eterowych w tkankach, komórkach i elementach subkomórkowych, a ponadto omawiają wpływ i znaczenie tych lipidów dla czynności organizmu. Metabolizm naturalnych lipidów eterowych zajmuje wiele miejsca w monografii. Szeroko zostały omówione dane dotyczące biosyntezy i rozpadu. Autor cytuje 271 prac zaczerpniętych z piśmiennictwa.

Wśród opracowań zajmujących się lipidami książka stanowi cenną pozycję i winna się znaleźć w bibliotekach biochemicznych.

Ryszard Niemiro

PHYSIOLOGY AND PATHOPHYSIOLOGY OF PLASMA PROTEIN METABOLISM. Proceedings of the International Symposium held in Stockholm, May 1967 (G. Birke, R. Norberg & L.-O. Plantin, eds.). Wenner-Gren Center International Symposium Series, vol. 12. Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig, 1969; str. 264, cena $ 12.50.

Książka stanowi zbiór referatów wygłoszonych w czasie czwartej z kolei konferencji z cyklu „Fizjologia i patofizjologia metabolizmu białek surowicy krwi." Ogromny rozwój badań w dziedzinie przemiany białek, jaki notuje się w ostatnich latach, skłonił organizatorów konferencji do ograniczenia tematu głównie do przemiany albumin i immunoglobulin. Wygłoszono 28 referatów, które przedstawione są w formie publikacji z całą dokumentacją i piśmiennictwem oraz dyskusją.

Tematycznie książka podzielona jest na pięć części. Część pierwszą — Rozdzielanie, oczyszczanie i znakowanie białek — zajmuje referat J. Killandera poświęcony badaniom własności chemicznych i metabolizmu znakowanego białka. Część druga — Kinetyka — zawiera trzy prace na temat metod badania metabolizmu znakowanych białek. Część trzecią — Regulacja i synteza białka — wypełnia dwanaście referatów omawiających syntezę, rozpad i regulację syntezy albumin oraz metabolizm immunoglobulin (IgG, IgA, IgM i IgD). Zespoły utraty białka to czwarta część książki. Składa się na nią osiem prac omawiających przemianę białek w niektórych jednostkach chorobowych. Ostatnia część — Różne — zawiera cztery referaty poświęcone przemianie witaminy B_{12}, przemianie lipoproteidów i roli albumin i globulin w ciśnieniu koloidalno-osmotycznym w przypadkach oparzeń.

Bardzo ciekawą częścią książki jest przytoczona dosłownie żywa i wnikliwa dyskusja. Dyskusja ta, w której uczestniczyli tacy specjaliści w tej dziedzinie jak Donato, Garby, McFarlane, Andersen, Nosslin, Freeman, Vetter, Killander i Plantin, obrazuje różnice w interpretacji obserwowanych faktów i daje jasny obraz współczesnej wiedzy o metabolizmie białek.

Tom zdobi fotografia uczestników Konferencji.

Ina Gąsiorowska

F. Franzen and K. Eysell, BIOLOGICALLY ACTIVE AMINES FOUND IN MAN. Their Biochemistry, Pharmacology and Pathophysiological Importance. Pergamon Press, Oxford 1969; str. 244, cena 100/— s., $ 13.50.

Od czasu ostatniej publikacji zbiorczej M. Guggenheima z 1951 r. przybyło wiele informacji na temat metabolizmu, zaburzeń, wzajemnego oddziaływania i stanów fizjologicznych i patofizjologicznych związanych z przemianami amin biogennych w ustrojach żywych. Recenzowana książka krótko i bardzo współcześnie wprowadza czytelnika w skomplikowane i nie całkiem jeszcze wyjaśnione mechanizmy działań aktywnych dla człowieka amin, jak tyramina, katecholoaminy, serotonina i histamina.

W części pierwszej podano zasady i schematy biosyntezy i rozpadu biologicznie aktywnych amin, enzymów uczestniczących w tych przemianych oraz aspekty fizjologiczne i patologiczne tych przemian. Druga część relacjonuje krótko własności farmakologiczne amin biogennych, ich rolę fizjologiczną jako przenośników w układzie nerwowym, wpływ na ciśnienie krwi, funkcje nerek i innych organów. Ostatnia część jest poświęcona znaczeniu omawianych amin w procesach patofizjologicznych, zaburzeniach neurohormonalnych oraz w szeregu jednostek klinicznych, jak phaeochromocytoma, guzy układu nerwowego (ganglioneuroma i neuroblastoma, ganglioneuroblastoma i in.).

Obszerne piśmiennictwo (1890 pozycji), cytowane w ostatnim rozdziale, relacjonuje wykrycie wielu różnorodnych amin w stanach patologicznych oraz patologicznych odchyleń przemian amin biogennych. Powyższy rozdział stanowi inteligentnie zebraną kompilację obszernej literatury klinicznej i badań laboratoryjnych z tego zakresu, przydatną zarówno dla lekarzy-praktyków, jak i analityków wdrażających nowe badania diagnostyczne w laboratoriach przyszpitalnych. Dla biochemików zaś duża ilość pochodnych amin biogennych wykrytych w stanach nieprawidłowych przemian jest wielce interesującym dowodem możliwości produkcyjnych ustrojów żywych — zmienionych w wyniku zaburzeń wywołanych chorobami. Śledzenie różnorakich bocznych łańcuchów przemian oraz procesów enzymatycznych nasilających te przemiany stanowi również miłą gimnastykę intelektualną.

Natomiast wadą tego rozdziału jest spojrzenie na zaburzenia patofizjologiczne ustroju ograniczone niemal wyłącznie do zmian biochemicznych metabolitów, próby doszukiwania się przyczyn wielu różnych schorzeń tylko na podstawie wyników laboratoryjnych oraz nieco mechanicystyczne spojrzenie na całość procesów zwanych odchyleniem od normy fizjologicznej. Nie jest to winą autorów, którzy niesłychanie sumiennie zebrali i przedstawili tak obszerne piśmiennictwo z zakresu amin biogennych występujących w ustroju człowieka, lecz chyba wypadkową spojrzenia większości badaczy zajmujących się tą dziedziną biochemii i farmakologii.

W konkluzji — książka zawierająca tyle odnośników źródłowych oraz doskonale zestawionych tablic zbiorczych winna znajdować się w rękach każdego, kto zajmuje się lub ma zamiar zapoznać się z obszerną i nie zbadaną jeszcze dziedziną biologicznie aktywnych amin w ustroju żywym.

Janusz Wysokowski