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TRANSAMINATION OF AROMATIC AMINO ACIDS IN RAT LIVER AND BRAIN

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Separation of the activities towards tyrosine, phenylalanine and tryptophan in rat liver mitochondria and rat liver and brain 30 000 *g* supernatants, was not achieved. However, the presented indirect evidence suggests that distinct enzymic activities might be involved. On purification of the enzyme, the activity ratio towards particular amino acids changed. Different activity ratios towards individual aromatic amino acids were also observed in the subcellular liver fractions and in brain supernatant. Distinct differences were found in the enzyme-coenzyme binding in respect to individual transamination activities and to their subcellular localization in liver mitochondria and in liver and brain supernatants.

So far, neither specificity of the transamination system of aromatic amino acids has been definitively elucidated, nor distribution of this enzymic activity in subcellular fractions has been unequivocally established. Canellakis & Cohen (1956), Jacoby & La Du (1964) and George & Gabay (1968) believe that transamination of aromatic amino acids is catalysed by a single enzyme showing broad substrate specificity. On the other hand, Kenney (1959) and Fonnum, Haavaldsen & Tangen (1964) postulate that distinct enzymes are involved in transamination of different aromatic amino acids. Kenney (1962) and Litwack, Sears & Diamondstone (1963), on the basis of experiments on rat liver concluded that tyrosine aminotransferase is located mainly in the soluble fraction of the cell, whereas Fellman, Vanbellinghen, Jones & Koler (1969) reported that the activity of this enzyme in mitochondria was about twice as high as in the supernatant fraction.

It is important to gain better insight into functioning of transamination system of aromatic amino acids because of its role in metabolism and hormonal control exerted upon this process in mammals.

In the present work, transamination of tyrosine, phenylalanine, 3,4-dihydroxyphenylalanine (DOPA) and tryptophan has been examined in rat liver mito-

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chondria and in the 30 000 *g* supernatant of rat liver and brain. An attempt was made to separate the enzymic activities catalysing transamination of particular amino acids. Interaction of aminotransferases with coenzyme, as well as the effect of parenteral administration of amino acids, were also studied.

A preliminary report of this work has been presented (Pużyńska & Mochnacka, 1968).

MATERIALS AND METHODS

Material. White male rats weighing about 140 - 160 g, kept on a standard diet, were used. They were fasted overnight and killed by decapitation, and brain and liver were isolated. All further steps of the procedure were carried out in the cold room at 4°C, and centrifugations were performed at 4°C in a cooled MSE centrifuge with a Super Speed rotor.

The tissue was homogenized in a Potter-type homogenizer with a polyacrylamide pestle at 600 rev./min in 4 volumes of 0.14 M-KCl, then centrifuged for 40 min at 30 000 *g*. The sediment was discarded and the supernatant used for the assays of enzymic activities.

Mitochondria were isolated from the 10% liver homogenate in 0.25 M-sucrose according to Schneider (1948): the 600 *g* supernatant was centrifuged at 10 000 *g* for 15 min, and the mitochondrial pellet was washed three times with 3 volumes of 0.25 M-sucrose. The mitochondrial fraction corresponding to 1 g of tissue was suspended in 1 ml of 50 mM-Na,K-phosphate buffer, pH 8, gently homogenized by hand, frozen at -15°C, and on the next day used for activity determinations.

Aminotransferase determination. The activity was assayed by the enol-borate tautomerase method according to Lin, Pitt, Civen & Knox (1958). The incubation mixture (2 ml) contained: 0.1 µmol of pyridoxal phosphate (PLP); enzyme source, 1 - 10 mg of protein; one of the following amino acids: L-tyrosine or 3,4-dihydroxyphenylalanine (DOPA), 6 µmol; L-tryptophan, 60 µmol; L-phenylalanine, 50 µmol; and Na,K-phosphate buffer, pH 8, at a final concentration of 0.2 M. After 5 min of preincubation, the reaction was started by addition of 40 µmol of α-oxoglutarate. α-Oxoglutarate at this concentration (20 mM) had no inhibitory effect, at variance with the results of Canellakis & Cohen (1956) and Tangen, Fonnum & Haavaldsen (1965). The incubation was carried out at 37°C, the mixture was then deproteinized with 0.5 ml of 20% metaphosphoric acid, and the respective enol-borate tautomers of the aromatic α-oxoacid formed were determined spectrophotometrically.

The extinction was read at the wavelengths given by Jacoby & La Du (1964): for *p*-hydroxyphenylpyruvate at 310 nm, $\varepsilon=12\,400$; for phenylpyruvate at 300 nm, $\varepsilon=9150$; for indolepyruvate at 332 nm, $\varepsilon=12\,700$; and for 3,4-dihydroxyphenylpyruvate at 340 nm, $\varepsilon=7700$.

The control samples in the assay of tyrosine and phenylalanine transamination contained no amino acid, and those in the assay of tryptophan transamination, were devoid of α-oxoglutarate. In the blank samples, α-oxoglutarate was added after deproteinization.

For determination of aminotransferase activity towards tyrosine, the method

of Diamondstone (1966) was also employed. The results of activity measurements obtained by this method were practically the same as those obtained by the enol-borate tautomerase method. However, the method of Diamondstone is twice as sensitive, and therefore it was used for the assay of the activity in brain, where the tyrosine transamination reaction is very low. Moreover, this method was used for the assays carried out in the presence of very large concentration of PLP (500 μM) as this compound interferes at higher concentrations with determinations by the tautomerase method (Bełżecka, Laskowska & Mochnacka, 1962; Hayashi, Granner & Tomkins, 1967).

In the method of Diamondstone, the standard incubation mixture contained in 3.2 ml of 0.2 M-Na,K-phosphate buffer, pH 8, 19.2 μmol of L-tyrosine, 0.16 μmol of PLP and 0.2 - 1.5 mg of protein. After 5 min, 32 μmol of α-oxoglutarate was added and the mixture was incubated at 37°C. When a fresh enzymic preparation was used, 0.2 ml of aqueous solution of 0.06 M-diethyldithiocarbamate (DDC) was added to the incubation mixture to inhibit the enzymic degradation of *p*-hydroxyphenylpyruvate. DDC was not added when the enzymic preparation had been stored frozen for a period longer than 36 h, as the enzymes catalysing this degradation are known to lose their activity within that period (Litwack, 1957); nor was DDC added in the assays by the enol-borate tautomerase method.

The reaction was stopped after 15 - 30 min by adding 0.2 ml of 10 N-NaOH, with vigorous stirring, and after 30 min at room temperature the extinction of *p*-hydroxybenzaldehyde (ε=19 000) was read at 331 nm against a blank sample prepared by adding NaOH to the incubation mixture prior to the addition of α-oxoglutarate.

In the assays by both methods the amount of protein and the time of incubation were chosen in such a way as to assure a linear reaction rate. The activities are expressed in nmol/min/mg protein.

Administration of amino acids, pyridoxine and hydrazine. The compounds to be administered were dissolved in physiological saline, neutralized if necessary, and injected intraperitoneally. A 7% solution of the appropriate amino acid was injected in a single dose corresponding to 0.7 mmol per 100 g body weight. Pyridoxine hydrochloride, 30, 100 or 200 mg per 100 g, was applied in one or two doses. The amount of applied hydrazine dihydrochloride was 30 mg per 100 g body weight.

At different time intervals after the injections, up to 4 h, the animals were killed and the activity of aminotransferases of aromatic amino acids was assayed. The control animals were injected with physiological saline. It was found in preliminary experiments that administration of saline did not affect the studied enzymic activities. Also after the injection the animals received no food.

Protein was determined by the tannin micromethod of Mejbaum-Katzenellenbogen (1955). In the course of purification of the enzymes, protein was determined also by the spectrophotometric method of Warburg & Christian (1941).

Reagents. α-Oxoglutaric acid (Politechnika Śląska, Gliwice, Poland); sodium pyruvate, L-glutamic acid, and hydrazine dihydrochloride (Xenon, Łódź, Poland); pyridoxine hydrochloride (Krakowskie Zakłady Farmaceutyczne, Kraków, Poland);

L-tyrosine, β-alanine, diethyldithiocarbamate, glutathione (reduced), sodium dodecyl sulphate, and cethyl trimethyl ammonium bromide (British Drug Houses, Poole, Dorset, England); pyridoxal phosphate and DL-serine (Fluka A. G., Buchs, Switzerland); L-phenylalanine (Carlo Erba, Milano, Italy); 3,4-dihydroxyphenylalanine, *p*-hydroxyphenylpyruvic acid, 2-mercaptoethanol, sodium deoxycholate (Koch-Light Lab., Colnbrook, Bucks., England); L-cysteine (Schuchardt, Munich, G.F.R.); L-tryptophan and L-aspartic acid (Reanal, Budapest, Hungary); DL-α-alanine and L-histidine (Chemapol, Prague, Czechoslovakia); Triton X-100 (Rohm & Haas, Philadelphia, Pa., U.S.A.); Sephadex G-100 (Pharmacia, Uppsala, Sweden); Ecteola-cellulose, ET 11 Whatman column chromedia (Balston, Maidstone, Kent, England). Hydroxyapatite was prepared as described by Levin (1962).

RESULTS

Distribution of activity, pH *optima and* K_m *values*

The aminotransferase activities towards particular aromatic amino acids varied in the subcellular fractions of rat liver (Table 1). In the 30 000 *g* supernatant the activity, calculated per 1 mg of protein, was the highest towards tyrosine and much lower towards phenylalanine, whereas in the mitochondrial fraction transamination of phenylalanine was three times higher than that of tyrosine. Transamination of tryptophan and DOPA was higher in the mitochondrial fraction than in the supernatant. However, the sum of transamination activity was in these two fractions practically the same.

Table 1

Transamination activity in the 30 000 g *supernatant and mitochondrial fraction of liver, and 30 000* g *supernatant of rat brain*

Aminotransferase activity was determined by the enol-borate tautomerase method as described in Methods. The results are mean values of 5 - 8 experiments with liver, and 10 experiments with brain, limit values being given in parentheses.

Substrate	Transamination activity (nmol/mg protein/min)		
	Liver		Brain
	Supernatant	Mitochondria	Supernatant
L-Tyrosine (3 mM)	22.0 (18.0 - 27.0)	5.6 (5.0 - 6.5)	0.7* (0.5 - 0.9)
L-Phenylalanine (25 mM)	3.1 (2.5 - 4.0)	15.0 (14.0 - 16.0)	0.9 (0.7 - 1.2)
L-Tryptophan (30 mM)	1.8 (1.3 - 2.4)	8.0 (7.0 - 10.0)	0.8 (0.7 - 1.0)
L-DOPA (3 mM)	1.4 (1.2 - 1.5)	3.2 (2.7 - 3.6)	—

* Determination by the method of Diamondstone (1966) in the presence of 6 mM-tyrosine and 10 mM-α-oxoglutarate.

In rat brain supernatant, the transamination activity was much lower than that observed with liver and it was the same with all three aromatic amino acids.

The stability of the studied enzymic activities was rather high; the liver preparations on storage at -15°C retained full activity for two weeks. Neither was the activity in liver and brain supernatants decreased by three or four freezing-and-thawing cycles.

A broad pH optimum at 7.6 - 8.3 was observed in the liver mitochondrial preparation with all three studied aminotransferase activities (Fig. 1).

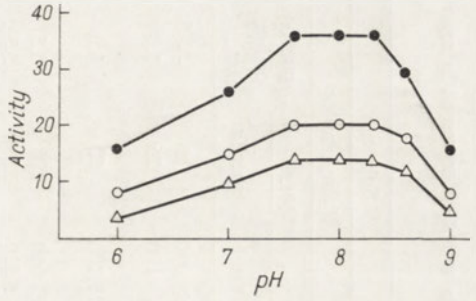

Fig. 1. Effect of pH on aminotransferase activity towards: ●, phenylalanine; ○, tryptophan, and △, tyrosine, in rat liver mitochondria. The activity was determined by the enol-borate tautomerase method as described in Methods, and expressed in nmol/min/sample. Protein, 2.5 mg/sample. In the pH range 6.0 - 8.3, 0.2 M-Na,K-phosphate buffer was used, and for pH 7.5 - 9.0, 0.2 M-tris-HCl buffer.

Table 2

Michaelis constants for transaminatcn of tyrosine, phenylalanine and tryptophan in liver supernatant and mitochondria and brain supernatant

The activity was determined by the enol-borate tautomerase method, with 20 mM-α-oxoglutarate.

Substrate	K_m (mM)		
	Liver		Brain
	Supernatant	Mitochondria	Supernatant
L-Tyrosine	1.7	2.0	3 - 5*
L-Phenylalanine	60 - 82	60 - 80	50 - 71
L-Tryptophan	33	30	33

* Determined by the method of Diamondstone (1966).

K_m values calculated from the Lineweaver-Burk plots are presented in Table 2. The affinity towards three amino acids was different; the lowest K_m values were found for tyrosine, about 2 mM, fifteen times higher values for tryptophan, and forty times higher for phenylalanine, but there was practically no difference in the affinity towards a given substrate in the mitochondrial and supernatant liver preparations and brain supernatant.

Table 3

Purification of the transamination system of aromatic amino acids from rat liver mitochondria

The mitochondria were disintegrated by four cycles of freezing and thawing. The activity is expressed in nmol/mg of protein/min.

Stages	Volume (ml)	Total protein (mg)	Substrate								
			Tyrosine			Phenylalanine			Tryptophan		
			activity		purification factor	activity		purification factor	activity		purification factor
			total	specific		total	specific		total	specific	
Mitochondrial suspension	60	1800	9540	5.3	—	27 000	15	—	14 400	8	—
80 000 *g* supernatant	50	850	7060	8.3	1.6	22 100	26	1.7	13 600	16	2.0
Ppt. at 0.4 - 0.75 $(NH_4)_2SO_4$ sat.	8	320	5440	17	3.2	19 200	60	4.0	8 960	28	3.5
Heating at 55°C (3 min) and dialysis	6	138	4690	34	6.4	17 660	128	8.5	6 070	44	5.5
Ecteola-cellulose	12	24	1680	70	13.2	5 520	230	15.3	2 640	110	13.7
Ppt. at 0.75 $(NH_4)_2SO_4$ sat.	3	18	1530	85	16.0	4 680	260	17.3	2 520	140	17.5
Sephadex G-100	3	4.2	504	120	22.6	2 230	530	35.3	1 510	360	45.0

Purification studies

The procedure of purification of liver mitochondrial enzyme is summarized in Table 3. The aminotransferase activity was tightly bound to the mitochondrial membrane: freezing of the mitochondrial suspension at -15°C for 12 h resulted in a release into the 10 000 *g* or 80 000 *g* supernatant of about a half only of the total activity. Treatment with a number of detergents or sonication did not lead to better solubilization. The best results were obtained by four cycles of freezing in an ethanol-solid CO_2 mixture and thawing.

Further stages of purification were carried out in cold room at 4°C, in the presence of 1 mM-EDTA and 1 mM-2-mercaptoethanol.

The 80 000 *g* supernatant of the disintegrated mitochondria was fractionated with ammonium sulphate at pH 8. All three activities were found in the protein fraction precipitating at 0.4 - 0.75 ammonium sulphate saturation. This protein fraction was dissolved in 1 mM-EDTA - 1 mM-2-mercaptoethanol - 5 mM-Na,K-phosphate buffer, pH 8, which contained 10 mM-α-oxoglutarate. The mixture was heated for 3 min at 55°C and cooled rapidly. The precipitated protein was centrifuged off, and the supernatant dialysed against 800 volumes of EDTA - mercaptoethanol - 5 mM-Na,K-phosphate buffer, pH 8, for 18 h. Then 4 ml of the solution (92 mg of protein) was submitted to chromatography on Ecteola-cellulose column (Fig. 2).

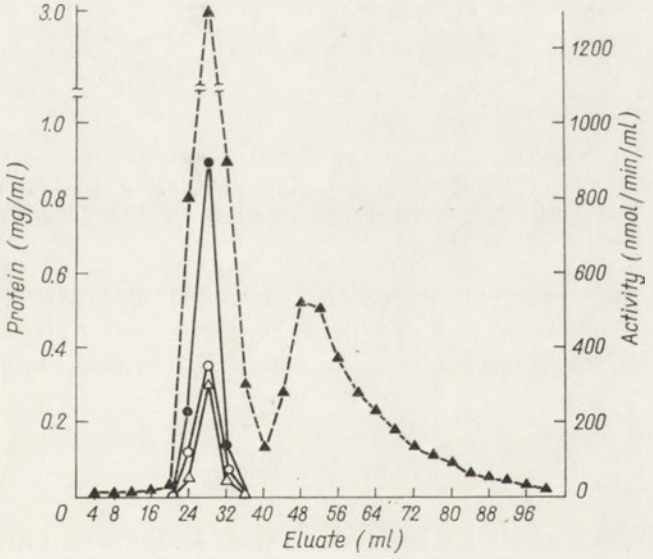

Fig. 2. Chromatography on Ecteola-cellulose column of liver mitochondria proteins precipitated at 0.4 - 0.75 ammonium sulphate saturation. To the column (2×25 cm) equilibrated with 5 mM-Na,K-phosphate buffer, pH 8, 92 mg of protein was applied and eluted with the same buffer, fractions of 4 ml being collected. Protein (- - ▲ - -) was determined spectrophotometrically, and the aminotransferase activity towards ●, phenylalanine; ○, tryptophan and △, tyrosine, by the enol-borate tautomerase method.

Two protein peaks were obtained, of which the first one contained the aminotransferase activity towards all three substrates. The active fractions of the eluate (20 - 32 ml) were pooled and ammonium sulphate was added to 0.75 saturation. The precipitated protein was dissolved in 3 ml of EDTA - mercaptoethanol - 50 mM-Na,K-phosphate buffer, pH 8, and passed through a Sephadex G-100 column (Fig. 3). The applied procedure gave a 23-fold purification of the activity towards

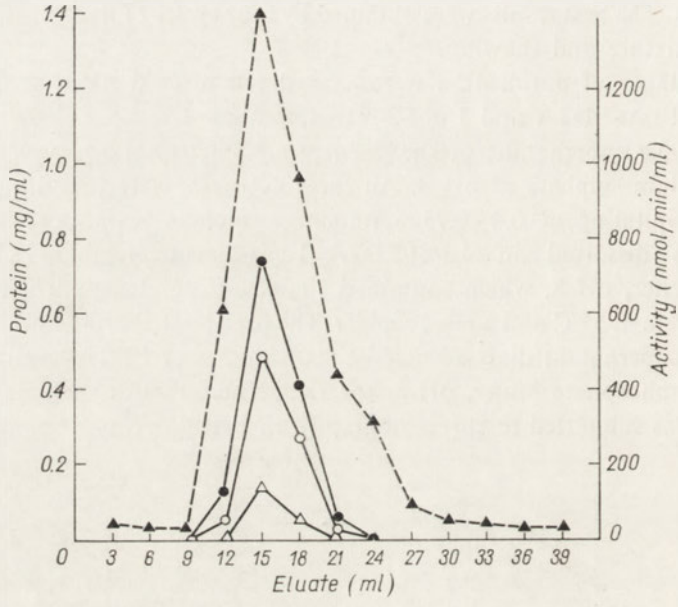

Fig. 3. Sephadex G-100 gel filtration of the active peak from Ecteola-cellulose chromatography (see Fig. 2). To the column (1.5×20 cm), 16 mg of protein was applied and eluted with 50 mM-Na, K-phosphate buffer, pH 8.0, fractions of 3 ml being collected. Other details as in Fig. 2.

tyrosine, 35-fold towards phenylalanine and 45-fold towards tryptophan, the corresponding yields being 5, 8 and 10%. The three studied activities were not separated by the applied procedure, nor were they separated on a column of hydroxyapatite.

Interaction with coenzyme

In experiments *in vitro* it has been found (Table 4) that in the supernatant from rat liver the aminotransferase system was not saturated with coenzyme. Addition of PLP to a concentration of 50 μM in the reaction mixture, led to a fourfold increase of the enzyme activity towards tyrosine and threefold towards phenylalanine and tryptophan. On the other hand, in liver mitochondria (both fresh and frozen) or brain supernatant, the addition of PLP, even at a concentration of 500 μM, had no effect (in the latter case the determinations were made by the method of Diamondstone

in view of the high concentration of PLP). This indicates that the aminotransferases of liver mitochondria and brain supernatant were saturated with coenzyme.

To remove PLP from the enzyme *in vitro*, the procedure used by Klein & Sagers (1966) for studying the glycine decarboxylation system, was applied; it consists in incubating the enzyme preparation with cysteine and prolonged dialysis. In liver supernatant, this treatment caused a considerable decrease in enzyme activity towards tyrosine but had no effect on transamination of phenylalanine or tryptophan. The addition of PLP enhanced the activity, especially towards tyrosine, but it did not lead to values as high as those obtained before treatment with cysteine.

When brain supernatant was treated with cysteine and dialysed, dissociation of coenzyme from the enzymic protein was not observed; this suggests very tight binding of coenzyme by the brain enzyme(s).

To remove the coenzyme *in vivo*, rats were injected intraperitoneally with hydrazine (Killam & Bain, 1957). Hydrazine, 30 mg/100 g, after 70 min lowered the activity in liver supernatant and mitochondria but did not affect the activity in brain supernatant (Table 4).

The effect of hydrazine administration may indicate that, although both in brain supernatant and in liver mitochondria the enzymes are saturated with coenzyme, the bond with PLP in the mitochondria is more labile. This assumption was supported by the fact that severalfold freezing and thawing of the liver mitochondrial fraction led to some decrease in transamination activity, which could be restored by the addition of PLP. Severalfold freezing of the brain supernatant did not result in dissociation of coenzyme.

Parenteral administration to rats of a single dose of 30 mg of pyridoxine had no effect on the transamination activity in liver supernatant but higher doses, 150 - 200 mg, were effective. Administration of 2×100 mg caused after 4 hours a marked increase of the activity towards tyrosine in comparison with the control value, both in the presence and absence of added PLP (Table 4). Simultaneously an increase of the enzyme activity towards phenylalanine and tryptophan was observed, which was enhanced by the addition of PLP although to a lesser extent than the activity towards tyrosine.

These results show that administration of pyridoxine leads both to a greater saturation of the enzyme with coenzyme in liver supernatant, and to an increase of the population of enzymic protein molecules, the enzyme still being "unsaturated" with coenzyme.

In liver mitochondria and in brain supernatant administration of pyridoxine had no effect on the aminotransferase activities assayed either with or without the addition of PLP. Nor did parenteral administration of 10 mg of PLP alter the activity of brain supernatant assayed after 90 min. These results are in agreement with those obtained *in vitro* and show that in liver mitochondria and brain supernatant the enzymic proteins are saturated with coenzyme.

Table 4

The effect of PLP and cysteine-dialysis treatment in vitro, *and pyridoxine and hydrazine administration* in vivo, *on the transamination activities*

The enzymic preparations were treated with cysteine and dialysed as described in Methods. Hydrazine was administered intraperitoneally, 30 mg/100 g body weight, and after 70 min the activity was determined. Pyridoxine was administered in 2 doses of 100 mg/100 g body weight, and after 4 h the activity was determined.

The results are mean values from 3 - 4 experiments and for control rats from 5 - 7 experiments. The activity was determined by the enol-borate tautomerase method as described in Methods, without (—) and with (+) the addition of PLP at a final concentration of 50 μM. The activity is expressed in nmol/mg of protein/min.

Source of enzyme	Substrate	Control		Removal of PLP				Pyridoxine administration	
				in vitro (cysteine and dialysis)		*in vivo* (hydrazine administration)			
		+PLP	—PLP	+PLP	—PLP	+PLP	—PLP	+PLP	—PLP
Liver, 30 000 *g* supernatant	Tyrosine	25.0	6.0	11.1	0.8	9.6	4.0	94.0	43.0
	Phenylalanine	3.1	0.9	2.0	0.7	1.2	0.5	4.8	4.0
	Tryptophan	1.8	0.6	1.0	0.5	0.7	0.4	2.5	2.0
Liver, mitochondria	Tyrosine	5.6	5.3	—	—	4.1	3.2	6.4	5.2
	Phenylalanine	16.0	15.9	—	—	12.7	9.0	14.0	11.1
	Tryptophan	9.4	9.3	—	—	6.6	5.1	9.8	7.5
Brain, 30 000 *g* supernatant	Tyrosine*	0.7	0.7	1.0	0.8	0.7	0.6	0.8	0.8
	Phenylalanine	0.9	0.8	1.1	1.0	0.9	0.7	0.9	0.7
	Tryptophan	0.8	0.9	1.0	0.9	0.8	0.7	0.9	1.0

*Determined by the method of Diamondstone (1966).

Effect of parenteral administration of amino acids

The transamination activity in the 30 000 *g* supernatant of rat liver after administration of various amino acids, is presented in Table 5. The aromatic amino acids, aspartate and glutamate, as well as serine and alanine, induced to a greater or lesser extent transamination of aromatic amino acids, administration of histidine had no effect, whereas *β*-alanine enhanced only transamination of tyrosine and DOPA without influencing that of phenylalanine or tryptophan.

The increase in aminotransferase activity towards tyrosine and DOPA was the greatest, and similar in magnitude. Four hours after administration of an aromatic or acidic amino acid, the activity increased six- to tenfold. The activity towards phenylalanine increased after administration of various amino acids only two- to threefold, and towards tryptophan, two- to fourfold. This last activity was sevenfold specifically stimulated by administration of tryptophan. The effect of tyrosine and phenylalanine administration was determined after 2 and 4 hours. On tyrosine administration, an increase with time was observed, whereas no such effect was noted with phenylalanine.

In liver mitochondria and brain supernatant, no changes in transamination activity were observed after administration of amino acids.

DISCUSSION

The attempt to separate the enzymes catalysing transamination of individual aromatic amino acids in rat liver mitochondria, was unsuccessful. Similarly, Jacoby & La Du (1964) have not separated the activities in the 16 000 *g* supernatant of rat liver. On the other hand, Tangen *et al.* (1965) reported separation of tyrosine - *α*-oxoglutarate aminotransferase from tryptophan - oxaloacetate aminotransferase in rat brain supernatant.

Despite the failure in separating the discussed activities, some indirect evidence obtained indicates that different enzymes are involved in transamination of particular aromatic amino acids. Changes in the activity ratios towards amino acid substrates on purification of the enzymic preparation provide one of these indications. In the crude extract from liver mitochondria, the activity ratio towards tyrosine and phenylalanine was 1:2.8, and in the about 20-fold purified preparation, 1:4.4; the corresponding values for tyrosine and tryptophan were 1:1.5 in crude extract and 1:3.0 after purification.

Another indirect indication of the occurrence of three separate enzymes comes from the demonstrated differences in the degree of saturation and binding of enzymic protein with coenzyme when specific transamination reactions are assayed. In liver supernatant the activity towards tyrosine was more stimulated by addition of PLP to the reaction mixture, and considerably more by administration of pyridoxine *in vivo*, than the activity towards phenylalanine or tryptophan. This may indicate that the enzyme(s) active with phenylalanine and tryptophan is more saturated with

Table 5

The effect of amino acid administration on transamination activity towards aromatic amino acids in the 30 000 g supernatant of rat liver

At the indicated time after amino acid administration, the animals were killed and the transamination activity was determined as described in Methods. The results are mean values from 3 - 4 experiments (for the control from 5 - 8 experiments). The activity is expressed in nmol/mg of protein/min, limit values being given in parentheses; I.f, increasing factor.

Amino acid administered (0.7 mmol/100 g body wt.)	Time (h)	Substrate							
		Tyrosine	I.f.	Phenylalanine	I.f.	Tryptophan	I.f.	DOPA	I.f.
None (control)	—	22 (18 - 27)	—	3.1 (2.5 - 4)	—	1.8 (1.3 - 2.4)	—	1.4 (1.2 - 1.5)	—
L-Tyrosine	2	55 (51 - 65)	2.5	4.3 (3.1 - 5.3)	1.4	2.2 (1.8 - 2.6)	1.2	4.2 (4 - 5)	3.0
	4	220 (180 - 260)	10.0	6.8 (6.2 - 8)	2.2	8 (7 - 9.7)	4.4	13 (12 - 14)	9.3
L-Phenylalanine	2	132 (124 - 140)	6.0	7.4 (6.9 - 8.7)	2.4	4 (3 - 5.4)	2.2	8.4 (7 - 9)	6.0
	4	118 (115 - 121)	5.4	4.0 (3.7 - 5.3)	1.3	8 (7 - 9)	4.4	7 (6 - 8)	5.0
L-Tryptophan	4	190 (180 - 205)	8.6	9.3 (8.7 - 12)	3.0	13 (12 - 13.6)	7.2	4.5 (3 - 5)	3.2
L-Glutamate	4	153.8 (141 - 162)	7.0	6.5 (5.6 - 8.3)	2.1	5.2 (4.9 - 5.4)	2.9	8 (7.2 - 9)	5.7
L-Aspartate	4	135 (118 - 150)	6.1	11.8 (10.6 - 14)	3.8	6.4 (5.2 - 7.2)	3.6	14 (13 - 15)	10.0
DL-α-Alanine	4	66 (62 - 70)	3.0	5 (4.4 - 6)	1.6	3.4 (3 - 4)	1.9	5 (4.2 - 6)	3.6
DL-Serine	4	111 (100 - 130)	5.0	8.7 (8.1 - 10)	2.8	5 (4.5 - 5.3)	2.8	8.4 (7.6 - 9)	6.0
β-Alanine	4	55.6 (52 - 60)	2.5	3.1 (2.9 - 3.7)	0	2 (1.8 - 2.5)	1.1	3.4 (3 - 4)	2.4
L-Histidine	4	25 (20 - 30)	1.1	3.1 (2.5 - 4.7)	0	1.8 (1.5 - 2.4)	0	1.4 (1.3 - 1.5)	0

PLP. The effect of cysteine *in vitro* and hydrazine *in vivo* shows that these differences involve also strength of protein-to-coenzyme binding. The applied treatments removing PLP from the enzymic protein, had no effect on the activity towards phenylalanine and tryptophan but diminished considerably the activity towards tyrosine. Different response of the individual transamination activities has also been demonstrated in liver supernatant on intraperitoneal administration of amino acids Distinctly higher activity towards tyrosine as compared with the other activities, implies the existence of individual enzymic proteins.

A further proof for this supposition is the demonstration of the different activity ratios towards aromatic amino acids in subcellular liver fractions and brain supernatant, K_m values for a given aromatic amino acid being, however, practically the same. In the 30 000 *g* liver supernatant, this activity ratio towards tyrosine and phenylalanine was 1:0.14, in liver mitochondria 1:2.7, and in brain supernatant 1:1.3. These ratios for tyrosine and tryptophan were, respectively, 1:0.08, 1:1.4 and 1:1.14.

Differences in the enzyme-coenzyme binding are also observed on examination of the enzymes from individual subcellular fractions. Aminotransferases of liver mitochondria and brain supernatant, at variance with the enzyme(s) present in liver supernatant, were found to be saturated with coenzyme, and their activity was not enhanced by addition of PLP, nor was it affected by intraperitoneal administration of pyridoxine. Similarly, no stimulation by PLP of aromatic amino acid transamination has been observed by Miller & Litwack (1969) in rat brain mitochondria, by Mark, Pugge & Mandel (1970) in rat brain homogenate, and by George & Gabay (1968) in 900-fold purified preparation of phenylalanine - α-oxoglutarate aminotransferase from pig brain.

In our experiments, the aminotransferases present in liver mitochondria and brain supernatant were not affected by cysteine treatment *in vitro* or hydrazine administration *in vivo*, which indicates tight binding of coenzyme to enzyme protein in these preparations.

The results of the present work support the view that transamination of tyrosine, phenylalanine and tryptophan is catalysed by three distinct enzymes.

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TRANSAMINACJA AMINOKWASÓW AROMATYCZNYCH W WĄTROBIE I MÓZGU SZCZURA

Streszczenie

Badano aktywność aminotransferaz aminokwasów aromatycznych w mitochondriach wątroby oraz w 30 000 *g* supernatancie wątroby i mózgu szczura. Próby rozdzielenia aktywności względem tyrozyny, feniloalaniny i tryptofanu nie powiodły się. Otrzymano jednak pośrednie dowody, że mogą to być różne enzymy. Przy oczyszczaniu enzymu zmienia się stosunek aktywności względem poszczególnych aminokwasów; również wpływ fosforanu pirydoksalu *in vitro* i pirydoksyny *in vivo* jest różny. Ponadto wykazano różnice pomiędzy frakcjami subkomórkowymi i pomiędzy tkankami w nasyceniu białka enzymatycznego koenzymem i w sile wiązania koenzymu.

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CHARACTERISTICS OF ARGINASES FROM PLANT, UREOTELIC AND URICOTELIC ORGANISMS

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1. As representative arginases of plants, ureotelic and uricotelic animals, lupin, ox liver and chicken liver enzymes were studied. 2. The different effect of thiol group reagents on the enzymes may suggest that free thiol groups are essential for the activity of lupin arginase, and are not involved in the activity of ox liver arginase. Chicken liver enzyme was inhibited by these reagents only at their higher concentrations. 3. Approximate molecular weight of lupin arginase was found to be 150 000 as compared with 119 000 and 252 000, respectively, for ox liver and chicken liver enzymes. 4. The K_m values of the three arginases increased with increasing molecular weight.

Arginases obtained from liver of ureotelic and uricotelic animals show different capacity to hydrolyse L-arginine, different molecular weight (Mora, Tarrab, Martuscelli & Soberón, 1965b) and different types of inhibition by basic amino acids (Mora, Tarrab & Bojalil, 1966). It has also been demonstrated that the natural inhibitor of arginase isolated from sunflower seeds (nitrogen derivative of chlorogenic acid) is much more active towards arginase from bitter lupin seedlings than towards the enzyme from ox liver (Muszyńska & Reifer, 1968).

In the present paper a comparison is made of some properties of arginases from plants of bitter lupin, ox liver and chicken liver, as representatives, respectively, of plant, ureotelic and uricotelic organisms.

MATERIAL AND METHODS

Reagents. Inhibitor of plant arginase, the nitrogen derivative of chlorogenic acid, was prepared from sunflower seeds as described by Muszyńska & Reifer (1970); L- and D-arginine were purchased from Reanal (Budapest, Hungary),

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chlorogenic acid and L-canavanine sulphate from Koch-Light Lab. (Colnbrook, Bucks., England), L-lysine and reduced glutathione from Schuchardt GmbH (Munich, G.F.R.), L-ornithine hydrochloride from E. Merck A. G. (Darmstadt, G.F.R.), *N*-ethylmaleimide and 2-mercaptoethanol from Sigma Chem. Co. (St. Louis, Mo., U.S.A.), Sephadex G-200 from Pharmacia (Uppsala, Sweden), γ-guanidinobutyrate, L-homoarginine hydrochloride and α-amino-β-guanidinopropionate hydrochloride from Calbiochem (Los Angeles, Calif., U.S.A.); α-amino-β-guanidinopropionate was a kind gift of dr. Zofia Porembska (Dept. of Biochemistry, Medical School, Warszawa). All other reagents were purchased from Fabryka Odczynników Chemicznych (Gliwice, Poland).

Enzyme preparations. Arginase from seven-day-old plants of blue bitter lupin was prepared as described by Ber, Muszyńska & Kleczkowski (1971). Arginase from chicken liver was prepared according to the method of Mora *et al.* (1965b), up to step 6 (precipitate at 50 - 60% ammonium sulphate saturation). Bovine liver arginase, 20 units/mg, was a preparation of Koch-Light Lab.

Arginase activity assay. Arginase preparation, 20 µl, in 0.02 M-Na,K-phosphate buffer, pH 7.2, was preincubated at 38°C with 25 µl of 0.01 M-$MnCl_2$ solution, pH 6.8 - 7.0. The amount of enzyme protein was 3 µg of lupin, 2.4 µg of ox liver, or 52 µg of chicken liver preparation. After preincubation for the time indicated below, 25 µl of 0.5 M-L-arginine solution (pH 10) and bidistilled water or TEA[1] buffer up to 150 µl was added (final pH 10) and the incubation was carried out at 38°C. The reaction was stopped by adding 50 µl of conc. $HClO_4$, the sample diluted with water to 2 ml and the urea formed determined by the method of Archibald as modified by Ratner (1955).

The arginase activity was studied under the optimum conditions of incubation and preincubation for each enzyme preparation. The time of preincubation was 60 min for lupin, 15 min for ox liver, and 10 min for chicken liver arginase. With the applied amount of enzyme protein and 83 mM-arginine, the reaction was linear with time up to 60 min for lupin arginase, and up to 120 min for the liver enzymes; the incubation time chosen for assays was 30 min for lupin, and 60 min for the liver enzymes.

The specific activities of the preparations under the above conditions, expressed as µmol of urea formed/mg protein/min, were 9 for lupin, 12 for ox liver and 0.4 for chicken liver arginase.

Molecular weight determination. The approximate molecular weights of arginases were estimated according to the method of Whitaker (1963). A Sephadex G-200 column (2.6×50 cm) was calibrated with bovine plasma albumin, grade A (Calbiochem), fumarase from pig heart, xanthine oxidase from milk (Koch-Light Lab.) and urease (Merck). The void volume of the column was established with Dextran blue 2000. About 10 mg of each protein in 1 ml of 0.05 M-tris buffer, pH 7.4, was

[1] Abbreviations used: pCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; TEA, triethanolamine.

applied on the top of the column equilibrated previously with the same buffer, and 1.7 ml fractions were collected, the rate of flow being 1 ml/200 sec. Protein in the eluate was estimated according to Lowry *et al.* (1951) and simultaneously the enzymic activities were determined.

RESULTS

The lupin arginase preparation was found to be less stable than the two liver arginases. The enzyme was completely inactivated by 7 h dialysis against 0.05 M-tris buffer, pH 7.4, in the presence of 1 mM-EDTA at 4 - 5°C, and could not be reactivated by the addition of Mn^{2+} ion to the incubation mixture. The presence of 5 mM-2-mercaptoethanol protected the enzyme during dialysis only partially, as Mn^{2+}, Ni^{2+}, Fe^{2+} or Fe^{3+} restored not more than 50% of the initial activity. The preparations of liver enzymes after dialysis against tris buffer containing only EDTA were fully reactivated by Mn^{2+} ion. Ni^{2+} and Co^{2+} ions had a smaller effect, whereas cadmium activated only ox liver arginase (Table 1).

Table 1

The effect of various ions on the activity of arginase preparations dialysed in the presence of EDTA

Lupin, ox liver or chicken liver preparation, 2 mg, was dissolved in 0.5 ml of 50 mM-tris-HCl buffer, pH 7.4, containing 1 mM-EDTA, and dialysed for 7 h at 4 - 5°C against 100 ml of the same buffer with EDTA, with constant stirring of the external fluid. The buffer was changed five times at intervals of 70 min. The metal ions were added as chlorides. The assays were carried out as described in Methods. The results are expressed as percentages of the activity before dialysis, taken as 100.

Enzyme preparation	Addition (mM)	Arginase from		
		lupin*	ox liver	chicken liver
Before dialysis	Mn^{2+}, 1.67	100	100	100
After dialysis	None	13	25	16
	Mn^{2+}, 1.67	57	100	86
	Ni^{2+}, 1.67	41	31	35
	Co^{2+}, 1.67	12	45	31
	Fe^{2+}, 1.67	51	19	11
	Fe^{3+}, 1.67	42	30	14
	Cd^{2+}, 1.67	9	39	8
	Cd^{2+}, 3.34	9	43	8
	Cd^{2+}, 6.68	9	74	8

* The preparation was dialysed with the addition of 5 mM-mercaptoethanol.

K_m values for L-arginine, calculated from the Lineweaver-Burk plots (Fig. 1) were 55, 29 and 200 mM, respectively, for the lupin, ox liver and chicken liver arginases. The findings with liver arginases were in good agreement with the results of Mora, Martuscelli, Ortiz-Pineda & Soberón (1965a). All three enzymes were

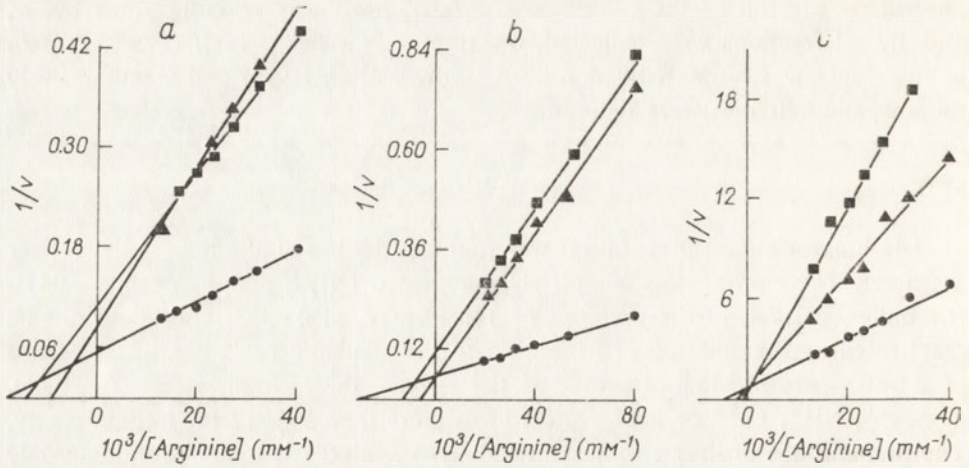

Fig. 1. Lineweaver-Burk plots for arginine hydrolysis by *a*, lupin, *b*, ox liver and *c*, chicken liver arginase preparations. ●, Uninhibited reaction; reaction with ▲, lysine, and ■, ornithine. The assays were performed as described in Methods with 25 mM-TEA buffer, pH 10; the final volume of the incubation mixture was 0.2 ml and arginine concentration 25 - 75 mM or 12.5 - 50 mM. The basic amino acids were used at the following concentrations: for lupin enzyme, 12.5 mM; for ox liver enzyme, 50 mM, and for chicken liver enzyme, 25 mM. The velocity, *v*, is expressed in μmol of urea formed/mg protein/min.

inhibited by lysine and ornithine. The experiments on the effect of various concentrations of basic amino acids (from 10 to 150 mM) showed that lupin arginase was affected to the greatest extent. The inhibition of lupin arginase by either of the two basic amino acids was of mixed type (Fig. 1a). In agreement with Mora *et al.* (1966), inhibition of chicken enzyme by these amino acids was of competitive type (Fig. 1b); with ox liver enzyme, lysine was a competitive inhibitor whereas inhibition by ornithine was of mixed type (Fig. 1c).

The effect of thiol group reagents on the three enzyme preparations is presented in Fig. 2. pCMB was found to be a very potent inhibitor of lupin arginase, as at

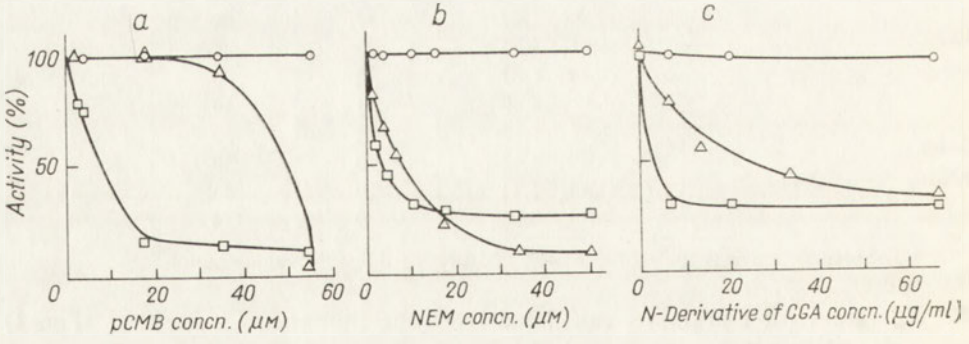

Fig. 2. The effect of *a*, *p*-chloromercuribenzoate, *b*, *N*-ethylmaleimide, and *c*, *N*-derivative of chlorogenic acid, on the activity of arginases from □, lupin; ○, ox liver; and △, chicken liver. The indicated amounts of the inhibitor were preincubated with the enzyme preparation.

15 μM concentration it caused a decrease in activity of 85%. This concentration of pCMB had no effect on chicken liver enzyme, but a concentration of 55 μM resulted in its complete inhibition. Ox liver arginase was not affected by the applied concentrations of pCMB (Fig. 2a), in agreement with the results of Mora *et al.* (1966) who demonstrated that 1 mM-pCMB had no effect on the activity of rat liver arginase and completely inhibited the activity of chicken liver arginase. NEM, similarly as pCMB, had no effect on the activity of ox liver arginase, whereas it inhibited both lupin and chicken liver arginases (Fig. 2b). *N*-Derivative of chlorogenic acid, the natural arginase inhibitor from sunflower seeds, practically did not change the activity of ox liver enzyme, inhibited to some extent the chicken liver enzyme, and had the greatest effect on lupin arginase (Fig. 2c). The inhibition of the chicken liver enzyme by the *N*-derivative of chlorogenic acid was of non-competitive type, similarly as it has been demonstrated for lupin enzyme by Muszyńska & Reifer (1970).

Chlorogenic acid at concentrations from 0.05 to 0.4 mM caused a decrease in the activities of all three arginases, but it had the smallest effect on ox liver enzyme (Fig. 3a). Iodoacetate at concentrations of 1 - 70 mM inhibited all three enzymes to a similar extent (Fig. 3b).

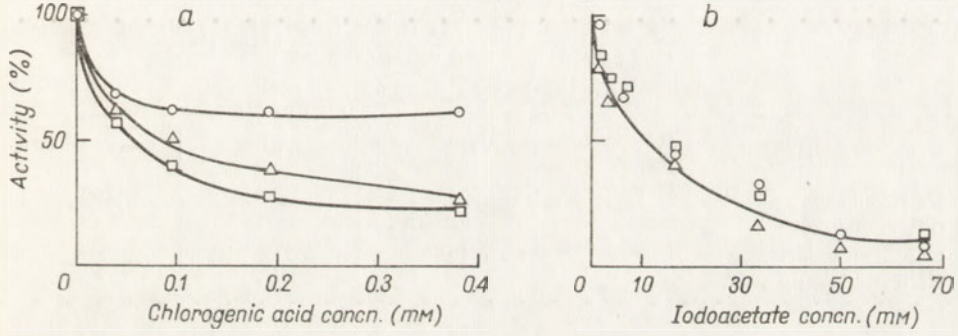

Fig. 3. The effect of *a*, chlorogenic acid, and *b*, iodoacetate on the activity of arginase from □, lupin; ○, ox liver; and △, chicken liver. The indicated amounts of the inhibitor were preincubated with the enzyme preparation.

The lupin arginase preparation was active also towards L-homoarginine and to some extent towards D-arginine (Table 2). Ox liver preparation had a very small activity towards D-arginine and L-homoarginine. Other guanidino compounds assayed were not hydrolysed in detectable amounts by these two preparations. Chicken liver preparation, in agreement with the results of Mora *et al.* (1965b), hydrolysed D-arginine and γ-guanidinobutyrate more efficiently than L-arginine. It showed also a slight activity towards L-homoarginine and L-canavanine.

The K_m values for the guanidino compounds hydrolysed by the arginase preparation : are shown in Table 3.

The approximate molecular weights determined by Sephadex gel filtration (Fig. 4) were 150 000 for lupin arginase, 119 000 for ox liver, and 252 000 for chicken liver arginases.

Table 2

Hydrolysis of guanidino compounds by lupin, ox liver and chicken liver arginase preparations

Conditions of assay as described in Methods, with 25 mM-TEA buffer, pH 10. The results are expressed as percentages of hydrolysis of L-arginine, taken as 100. BLD, below limits of detection.

Substrate (83 mM)	Arginase from		
	lupin	ox liver	chicken liver
L-Arginine	100	100	100
D-Arginine	10	7	191
L-Homoarginine	26	6	12
γ-Guanidinobutyrate	BLD	BLD	305
α-Amino-β-guanidinopropionate	BLD	BLD	BLD
L-Canavanine	BLD	BLD	9

Table 3

Michaelis constants of lupin, ox liver and chicken liver arginases for different guanidino compounds

Conditions of assay as described in Methods, with 25 mM-TEA buffer, pH 10; substrate concentration 25 - 250 mM, final volume 0.2 ml.

Substrate	K_m (mM) of arginase from		
	lupin	ox liver	chicken liver
L-Arginine	55	29	200
D-Arginine	—	—	42
L-Homoarginine	151	—	—
γ-Guanidinobutyrate	—	—	62

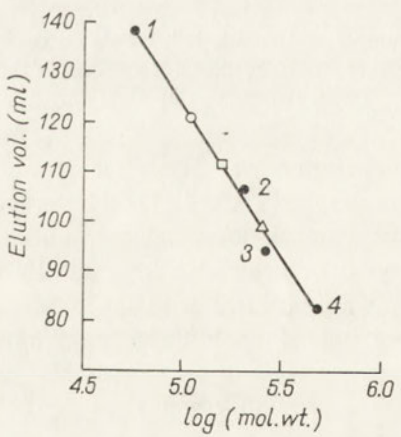

Fig. 4. Determination of approximate molecular weight of arginases from □, lupin; ○, ox liver; and △, chicken liver, by Sephadex G-200 gel filtration. Marker proteins: *1*, bovine serum albumin (mol. wt. 67 000); *2*, fumarase (mol. wt. 194 000); *3*, xanthine oxidase (mol. wt. 290 000); *4*, urease (mol. wt. 483 000). For details see Methods.

DISCUSSION

In the present work, some differences were found to exist between a plant arginase and animal "ureotelic" and "uricotelic" arginases. Lupin arginase appeared to be very unstable and removal of metal ions by dialysis in the presence of EDTA resulted in its complete inactivation. Partial protection of the enzyme during dialysis could be obtained by the addition of 2-mercaptoethanol. On the other hand, ox liver and chicken liver arginase activities after dialysis in the presence of EDTA were completely reactivated by addition of Mn^{2+} ions.

The lupin arginase, in contrast to the enzymes from ox liver and chicken liver, is characterized by a marked susceptibility to thiol group reagents, even when applied at very low concentrations. Muszyńska & Reifer (1970) have demonstrated that thiol compounds protect lupin arginase against inactivation by *N*-derivative of chlorogenic acid. These results may indicate that in lupin arginase thiol groups are essential for the enzyme activity. The arginase from chicken liver is inhibited by the thiol-group binding reagents to a much smaller extent than lupin arginase. The arginase from ox liver is not affected by these reagents. Thus thiol groups seem not to be involved in its activity.

Iodoacetate at high concentrations inhibited the activity of the three arginases to the same extent. Carboxymethylation of proteins by iodoacetate is not a specific reaction for detection of thiol groups, but this compound may combine with reactive groups of histidine, lysine and methionine over the pH range from 7 to 10 (Gurd, 1967). Therefore it seems possible to conclude that one of these amino acids is present in the active centre of all three studied arginases.

The molecular weight of lupin arginase was found to be 150 000. The corresponding values for the "ureotelic" enzyme are 138 000 (rat liver; Mora *et al.*, 1965b) and 119 000 (ox liver; present experiments), and for the "uricotelic" enzyme from chicken liver 276 000 (Mora *et al.*, 1965b) and 250 000 (present work). Hirsch-Kolb & Greenberg (1968) have demonstrated that rat liver arginase of molecular weight 118 000 is composed of four subunits, each with molecular weight of 30 000. Reddy & Campbell (1968) reported the occurrence in earthworm gut of an arginase with a molecular weight of about 27 000.

If we assume that other arginases are also composed of 27 000 or 30 000 mol. wt. subunits, then lupin arginase would be composed of 5 or 6 subunits, and "uricotelic" arginase of 8 - 10 subunits. As the "ureotelic" arginase has a higher affinity to arginine, it may be concluded that the structure consisting of 4 subunits is optimal for enzyme activity. The lower affinity to arginine of the "uricotelic" and plant arginases may be related to their higher molecular weight.

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CHARAKTERYSTYKA ARGINAZY ROŚLINNEJ ORAZ ARGINAZ Z ORGANIZMÓW UREOTELICZNYCH I URIKOTELICZNYCH

Streszczenie

1. Badano właściwości arginazy z siewek łubinu, arginazy z wątroby wołu i wątroby kurczęcia jako przedstawicieli arginaz: roślinnej i zwierząt ureo- i urikotelicznych.

2. Różnice we wpływie związków blokujących grupy SH wskazują, że grupy te są konieczne dla aktywności arginazy roślinnej, a nie są tak istotne dla obu arginaz wątrobowych.

3. Arginaza z łubinu ma ciężar cząsteczkowy około 150 000, natomiast arginaza z wątroby wołu i kurczęcia odpowiednio 119 000 i 252 000.

4. Wartości K_m badanych arginaz wzrastają wraz ze zwiększającym się ciężarem cząsteczkowym.

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THE INFLUENCE OF BASIC PROTEINS ON POLYSOMES. THE INTERACTION OF HISTONES WITH RAT LIVER POLYSOMES

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Sucrose gradient analysis proved that histones inhibit the activity of the cell-free protein biosynthetic system isolated from rat liver, by association with polysomes. Polysomes can be released without any apparent change from such associates by trypsin treatment. Histone *f1* has different affinity to polysomes than the remaining histones.

Histones are regarded as important factors involved in regulation of biosynthesis of nucleic acids and proteins within the cell nuclei (Huang & Bonner, 1962; Allfrey, Littau & Mirsky, 1963; Sluyser, Thung & Emnelot, 1965; Wang, 1966). There is also evidence that histones play a role in inhibition of protein biosynthesis in ribosomal systems *in vitro*. This effect has been demonstrated in systems isolated from rat liver (Berlinguet & Normand, 1968), rabbit reticulocytes (Kruh & Labie, 1968) and pea seedlings (Toczko, Sieliwanowicz & Jachymczyk, 1969). Several investigators have demonstrated that in the cell cytoplasm molecules of mRNA can exist as inactive ribonucleoprotein particles (Spirin, 1966, 1969; Henshaw, 1968) which can be activated by trypsin treatment (Monroy, Maggio & Rinaldi, 1965; Östner & Hultin, 1968; Jachymczyk, Zawierucha & Sieliwanowicz, 1971). These results suggested that in cytoplasm basic proteins may be involved in the regulation of protein biosynthesis.

Recently Jachymczyk & Sieliwanowicz (1971) have shown that the inhibitory effect of histones on the rat liver ribosomal fraction could be reversed almost completely by short treatment with trypsin. It was also found that addition of exogenous mRNA (or poly U) to the system containing the ribosomal fraction blocked by histones, resulted in an increase of the incorporation of radioactive amino acids into protein, whereas ribosomal RNA, with no template activity, had no such effect. Thus it could be supposed that histones cause inactivation of polysomes without affecting the activity of free ribosomes.

In the present experiments, the linear sucrose gradient analysis was performed

to obtain more data concerning the above-mentioned suggestion. The specificity of particular histones in their ability to associate with rat liver ribosomal preparations were also studied.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade. ATP, GTP, creatine phosphokinase and trypsin (2×cryst.) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Difco protein hydrolysate (vitamin-free casamino acids) from Difco Laboratories Inc. (Detroit, Mich., U.S.A.); creatine phosphate from Calbiochem (Los Angeles, Calif., U.S.A.); L-[U-^{14}C]leucine (spec. activity 311 mCi/mmol) and a mixture of ^{14}C-labelled amino acids (spec. act. 52 mCi/milliatom of carbon) from the Radiochemical Centre (Amersham, Bucks., England). Reagents for polyacrylamide gel electrophoresis were from Serva (Heidelberg, G.F.R.).

Preparation of histones. Total histones were prepared from calf thymus as described by Johns, Phillips, Simpson & Butler (1961). Histone preparations were analysed by the polyacrylamide disc electrophoresis technique (Johns, 1967, modified by Toczko, 1969) before being used in the experiments.

Preparation of ribosomal fraction and 105 000 g supernatant. These were prepared from liver of Wistar rats as described by Jachymczyk & Sieliwanowicz (1971). The concentration of ribosomes was determined by the method of Tester & Dure (1966) assuming $E_{260}=12$ to be equivalent to 1 mg of ribosomes.

The ribosomal preparation used for experiments contained 10 mg of ribosomes in 1 ml of 0.25 M-sucrose - 5 mM-$MgCl_2$ - 0.01 M-$KHCO_3$ - 0.025 M-tris-HCl buffer, pH 7.6.

Linear sucrose gradient analysis. A 0.2 ml sample containing a known quantity of ribosomes (usually 1 - 1.5 mg), native or treated with histones, was layered on a linear sucrose gradient from 10% to 34% (Jachymczyk & Cherry, 1968) containing 0.01 M-$MgCl_2$, 0.02 M-KCl, 0.01 M-tris-HCl buffer, pH 7.6, and 0.001 M-spermidine, and centrifuged at 35 000 rev./min in a Spinco SW 39 rotor for 60 min. To determine the distribution of ribosomes in the gradient, 45 - 50 fractions, 8 drops each, were collected from the bottom to the top of the gradient; samples were adjusted with water to a volume of 1.1 ml and extinction at 260 nm was read in Zeiss VSU-1 spectrophotometer (Jena, G.D.R.).

Assay of amino acid incorporation. The conditions for the incorporation of ^{14}C-labelled L-leucine were the same as described by Jachymczyk & Sieliwanowicz (1971).

Association of histones with ribosomal fraction. The ribosomal preparation, 0.1 ml (1 mg of ribosomes) was mixed with 1 ml of histone solution in 0.025 M-tris-HCl buffer, pH 7.4, and the mixture was made up to 2 ml with the 0.25 M-sucrose - 5 mM-$MgCl_2$ - 0.01 M-$KHCO_3$ - 0.025 M-tris-HCl solution of pH 7.6.

The obtained mixture was incubated with continuous stirring at 0°C for 10 min and centrifuged at 165 000 *g* for 60 min using Beckman ultracentrifuge model Spinco L-50. As a control, a parallel sample of ribosomes not treated with histones,

was centrifuged under the same conditions. The obtained pellets were analysed both for protein and RNA contents and the supernatant only for protein. RNA was determined according to Munro & Fleck (1966), proteins according to Lowry *et al.* (1951) using bovine serum albumin (Michrome, Gurr, London, England) as standard.

The protein in the supernatant was analysed also by polyacrylamide gel electrophoresis. The protein bands in gels were scanned using Zeiss densitometer ERJ-65 adapted for gel scanning.

RESULTS AND DISCUSSION

Effect of histones on ribosomal profiles. It has been observed that inhibition of the incorporation of radioactive L-amino acids into protein in rat liver ribosomal preparations by histones from pig renal cortex was dependent on the quantity of added histones (Jachymczyk & Sieliwanowicz, 1971). The same effect was found with calf thymus histones (Table 1). The addition of 100, 200 or 300 μg of histones per 1 mg of ribosomal fraction decreased the incorporation of amino acids into protein to 55%, 38% and 14%, respectively. When this effect of histones on ribosomal fraction was investigated by linear sucrose gradient analysis (Fig. 1), it was found

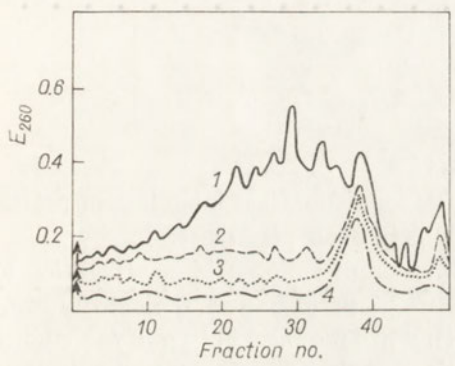

Fig. 1

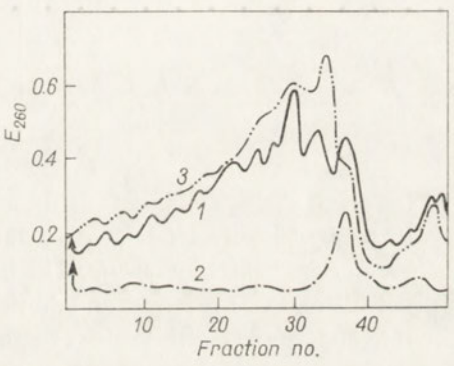

Fig. 2

Fig. 1. Effect of histones on the sucrose density gradient profiles of rat liver ribosome preparation. *1*, Intact ribosomes; ribosomes treated: *2*, with 100 μg histones/1 mg ribosomes; *3*, with 200 μg histones/1 mg ribosomes, and *4*, with 300 μg histones/1 mg ribosomes.

Fig. 2. Effect of trypsin on the restoration of polysomes. Sucrose gradient profiles of ribosomes: *1*, intact; *2*, treated with 300 μg histones/1 mg ribosomes; and *3*, treated with 300 μg histones and incubated with 2.7 μg of trypsin. Conditions for the trypsin incubation were the same as described in the legend to Table 1.

that histones reacted first with polysomes, forming with them complexes which precipitated and were spun down to the bottom of the tube (marked with arrow in Figs. 1 and 2). The addition of 100 μg of histones per 1 mg of ribosomes reduced the amount of polysomes by about 70% of that observed for the non-treated (control) ribosomal fraction. Under these conditions the amount of free ribosomes decreased only insignificantly. The addition of 200 or 300 μg of histones caused almost complete

precipitation of polysomes, whereas at least 50% of free ribosomes still remained in solution. Complete precipitation of free ribosomes occurred at much higher concentrations of histones ranging from 900 to 1000 μg per 1 mg of ribosomal fraction.

Table 1

The activity of rat liver ribosomes treated with histones, and the effect of trypsin

Ribosomes (1 mg in 0.1 ml) were mixed with histones dissolved previously in the same medium as ribosomes, made up to a final volume of 1 ml, kept at 0°C for 10 min, centrifuged, and used for the amino acid incorporation studies. In the experiment with trypsin, to the histone-ribosome mixture 0.05 ml of trypsin solution was added, and after 3 min at 37°C the reaction was stopped by cooling to 0°C and the mixture centrifuged at 165 000 *g* for 1 h.

The standard amino acid incorporation system (Jachymczyk & Sieliwanowicz, 1971) contained in 1 ml : 0.8 mg of 105 000 *g* supernatant protein (enzymic fraction), 0.4 mg of ribosomal fraction, and 0.5 μCi of L-[^{14}C]leucine. The data are the results of a typical experiment.

Addition (μg/mg ribosomes)		Incorporation (c.p.m./mg ribosomes)	Activity (%)
Histones	Trypsin		
None (control)	—	17 900	100
100	—	9 845	55
200	—	6 807	38
300	—	2 550	14
300	2.7	14 300	80

These observations confirmed the previous suggestion that inhibition of protein biosynthesis by histones is related to their action on polysomes.

Release of polysomes by trypsin. The inhibition of amino acid incorporation by 300 μg of histone was reversed on addition of 2.7 μg of trypsin, the activity being restored to 80% of the control value (Table 1). When the effect of trypsin was studied by sucrose gradient analysis (Fig. 2) it was found that trypsin released polysomes from the association with histones, as the profile of ribosomes treated with histones and then with trypsin resembled closely that of the native ribosomal preparation. The precipitate found at the bottom of the gradient disappeared after trypsin treatment, and the amount of polysomes in solution was even slightly higher than in the control.

It is thus evident that the inhibitory effect of histones cannot be connected with degradation of mRNA in polysomes, as in this case the release of polysomes by trypsin would not be possible. The previously observed influence of exogenous mRNA or poly U on the activity of histone-blocked ribosomal fraction (Jachymczyk & Sieliwanowicz, 1971) may be explained by low affinity of free ribosomes to histones. In fact, sucrose gradient analysis (cf. Fig. 1 and 2) showed that at least 50% of free ribosomes remained unchanged even after the addition of as much as 300 μg of histones per 1 mg of ribosomal fraction. These ribosomes should be able to form new polysomes.

The interaction of particular histones with the ribosomal fraction. The ribosomal fraction was incubated with various amounts of total histone preparation, and after centrifugation the non-bound histones in the supernatant were analysed by disc electrophoresis. The electrophoretograms of the supernatant obtained from the non-treated ribosomal fraction (Fig. 3A) and total histones from calf thymus (Fig. 3B) served as controls. When 300 μg of histones was added per 1 mg of ribosomal fraction, that is the amount of histones which inhibited almost completely the activity of the ribosomal fraction, no histones were found in the supernatant (Fig. 3C). Addition of 500 μg of histones resulted in the appearance in the supernatant

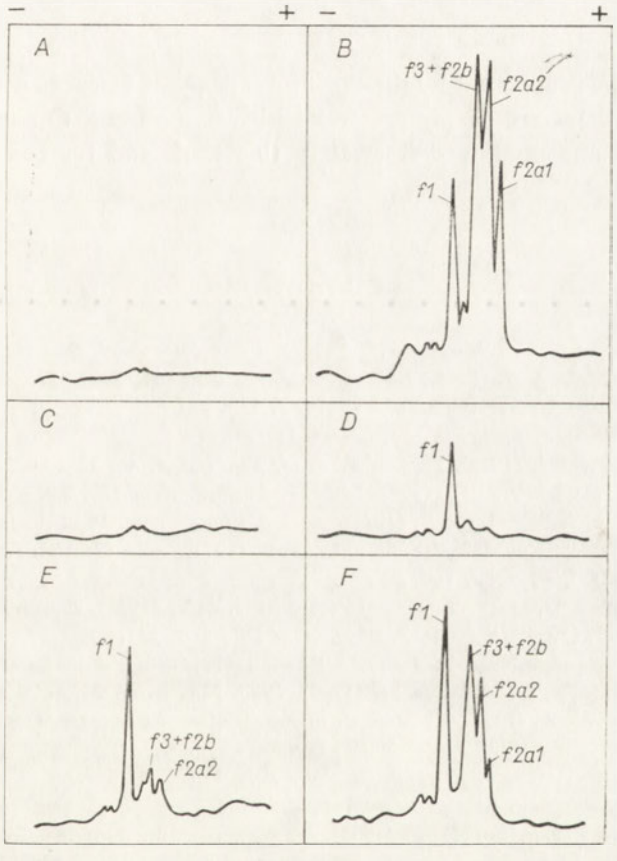

Fig. 3. The densitometer scans of gel electrophoretograms: *A*, supernatant of intact ribosomes; *B*, total histones; *C*, *D*, *E* and *F*, supernatants from ribosomes treated by, respectively, 300, 500, 700 and 1000 μg of total histones per 1 mg of ribosomes.

of histone *f1* (Fig. 3D), 700 μg, in the appearance also of histones *f3+f2b* and *f2a2* (Fig. 3E), and 1000 μg, of all kinds of histones. Thus, with the increasing amounts of histones, the differences in the affinity of particular histones to polysomes become clearly visible. This difference was especially notable with lysine-rich histone *f1*, and it could be due to the extended shape of this histone molecule (Haydon &

Peacocke, 1968), while other histones may form a more compact structure (Sluyser, 1969).

The capacity of polysomes to bind histones in much greater amount than it is necessary for complete inhibition of their activity suggests that the inhibition of protein biosynthesis caused by histones is connected with the blocking of only some specific places within polysomes. The presented data seem to contribute to the understanding of the mechanism of the inhibition by histones of protein biosynthesis in an *in vitro* system. It is very likely that basic proteins can participate in the regulation of translation in living cells by a mechanism similar to that observed in the cell-free system.

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WPŁYW BIAŁEK ZASADOWYCH NA POLISOMY. WZAJEMNE ODDZIAŁYWANIE HISTONÓW I POLISOMÓW WĄTROBY SZCZURA

Streszczenie

Za pomocą analizy w liniowym gradiencie stężeń sacharozy wykazano, że histony hamują aktywność bezkomórkowego układu do biosyntezy białka poprzez blokowanie polisomów. Polisomy mogą być uwalniane z połączeń z histonami pod wpływem trypsyny. Uwolnione polisomy, w liniowym gradiencie stężeń sacharozy nie różnią się od polisomów natywnych. Stwierdzono, że histon *f1* w porównaniu do pozostałych histonów wykazuje odmienną zdolność do łączenia się z frakcją rybosomalną.

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TRYPSIN INHIBITOR FROM BOVINE PITUITARY

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Isolation of the trypsin inhibitor from posterior lobe of bovine pituitary gland is presented. The inhibitor was purified by means of ammonium sulphate fractionation, DEAE- and CM-Sephadex chromatography and column electrophoresis. The isolated inhibitor was highly specific to trypsin and stable up to 60°C and at pH values 4 - 10. Optimum activity was at pH 6 - 10. The inhibitor reacts with trypsin at molar ratio 210 : 1 in the presence of a protein substrate, and 471 : 1 in the presence of *p*-tosyl-L-arginine methyl ester (TAME).

Studies carried out within the last ten years showed that proteolytic inhibitors are present in many animal tissues. In addition to the well-known pancreatic inhibitors, other proteolytic inhibitors were also found in brain, lungs and testicles (Fritz, Trautschold, Haendle & Werle, 1968; Sawaryn, 1969). Isolation of these compounds was facilitated by the recently introduced use of chromatographic columns filled with insoluble trypsin; this makes possible preparation of a highly purified inhibitor in one step.

The protein inhibitors, mainly trypsin inhibitors, inhibit specifically proteolytic activity by binding with the enzymes at 1 : 1 molar ratio. Only exceptionally other proportions have been found (Wagner & Riehm, 1967; Frattali & Steiner, 1968).

In the present paper, preparation and properties of trypsin inhibitor from posterior lobe of bovine pituitary is described. The isolated inhibitor is characterized by the unusually high molar excess with respect to trypsin.

MATERIALS AND METHODS

Chemicals. Sephadex G-25 (coarse), G-50 (fine), G-100, DEAE-Sephadex A-50, CM-Sephadex C-50 and Blue Dextran were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Trypsin, from bovine pancreas, 2 ×crystallized, dialysed and lyophilized; pepsin, 2 ×crystallized and lyophilized; papain from *Papaya Latex*, 2 ×crystallized; sodium acetate suspension; ribonuclease A from

bovine pancreas, 5 ×crystallized; α-casein and bovine serum albumin, crystallized and lyophilized; *p*-tosyl-L-arginine methyl ester (TAME), dithiothreitol and 2-mercaptoethanol were purchased from Sigma (St. Louis, Mo., U.S.A.). Chymotrypsin A, from bovine pancreas, crystallized, was obtained from Reanal (Budapest, Hungary). Pronase P, lyophilized, was purchased from Serva (Heidelberg, G.F.R.). All the other reagents used were analytical grade products. Whatman 3 *MM* chromatography paper was obtained from H. Reeve, Angel and Co. Ltd. (London, England), and visking dialysing tubing from Serva.

Determination of inhibitor activity: 1) Spectrophotometrically with casein as a trypsin substrate. To 0.2 ml of properly diluted inhibitor solution, 0.4 ml of 0.1 M-borate buffer, pH 8.0, containing 5 mM-$CaCl_2$, and 0.4 ml of trypsin were added (4 mg of trypsin was dissolved in 2 ml of 0.04 M-NaCl containing 0.02 M-$CaCl_2$; for the assay, 0.2 ml of this solution was diluted to 3 ml with borate buffer). After 10 min incubation at 37°C, 1 ml of casein (1% in borate buffer) was added and the reaction was carried out for 20 min. The reaction was stopped by the addition of 3 ml of 5% trichloroacetic acid. After 20 min the sample was filtered and extinction at 280 nm was measured.

2) Potentiometrically with casein as a trypsin substrate: 0.2 ml of trypsin solution (0.02% in 1 mM-HCl) and 0.1 ml of properly diluted inhibitor were added to 3.7 ml of 0.15 M-NaCl. After 10 min incubation at room temperature, 1 ml of casein (1%) was added and the reaction was measured with a pH-stat.

3) Potentiometrically with tosylarginine methyl ester (TAME) as a trypsin substrate: 0.1 ml of trypsin solution (0.02% in 1 mM-HCl) and 0.1 ml of properly diluted inhibitor were added to 3.8 ml of 0.15 M-NaCl. After 10 min incubation at room temperature, 1 ml of TAME solution (163 mg of TAME was dissolved in 2 ml of ethanol and adjusted to 5 ml with 0.04 M-NaCl containing 0.02 M-$CaCl_2$) was added, and the reaction was measured with a pH-stat.

The activity of inhibitor was expressed in terms of percentage of trypsin inhibition. One unit of inhibitor is defined as that amount of protein which under the test conditions inhibits 10% of trypsin activity.

Molecular weight was determined by gel filtration through Sephadex G-100 in 0.15 M-NaCl. To the column (1 × 100 cm), the mixture of Blue Dextran and 4-mg portions of inhibitor, albumin, pepsin and ribonuclease was applied and the elution was carried out with 0.15 M-NaCl. The elution volumes of standards and of the inhibitor were measured and molecular weight of the inhibitor was calculated from the graph: V_e/V_t *versus* log molecular weight.

High-voltage electrophoresis was carried out for 20 min in pyridine - acetic acid - water (10 : 0.4 : 89.6, by vol.) at pH 6.5 on Whatman 3 *MM* (10 × 57 cm) paper at 3.5 kV and 70 mA. After electrophoresis the paper was air-dried and sprayed with ninhydrin solution.

Preparation of insoluble trypsin. Insoluble trypsin was obtained in the reaction of the enzyme with the styrene - maleic anhydride interpolymer, in a way analogous to that used by Levin, Pecht, Goldstein & Katchalski (1964) for preparation of insoluble trypsin with ethylene - maleic anhydride copolymer.

Protein determination. Protein concentration in chromatographic fractions was monitored spectrophotometrically at 280 nm. Specific activity of the inhibitor was calculated basing on nitrogen content measured by the Kjeldahl method with ammonium chloride as standard.

RESULTS

Preparation and purification of the inhibitor. For preparation of the inhibitor, posterior lobes of frozen bovine pituitaries, obtained from slaughterhouse, were homogenized in Waring Blendor at 4°C with 4 volumes of water containing 10^{-5} M-EDTA. The obtained homogenate was stirred mechanically for 2 hours, pH was adjusted to 7, and stirring was continued for 30 min, followed by centrifugation at 6000 rev./min for 30 min. The extract showed high antitrypsin activity (9 units/mg N). An attempt to purify the inhibitor using insoluble trypsin was unsuccessful, therefore fractionation of the extract was performed as follows: to the supernatant, solid ammonium sulphate was added at pH 7 to 0.45 saturation and stirred for 1 hour. The precipitate was centrifuged off and discarded, and the supernatant was saturated with ammonium sulphate to 0.9 and left overnight with stirring. The pellet was dissolved in water and ammonium sulphate was removed by dialysis against water or by filtration through Sephadex G-25. Further purification was carried out by means of DEAE- and CM-Sephadex chromatography. DEAE-Sephadex (300 ml) was equilibrated with 0.01 M-succinate buffer at pH 6.5, the sample (600 mg) introduced, and the column was eluted successively with 0.01, 0.1 and 0.2 M-succinate buffer, pH 6.5 (Fig. 1). The obtained active material was fractionated on CM-Sephadex column (300 ml) equilibrated with 5 mM-ammonium acetate, pH 5.5. For the elution, 5 mM and 50 mM-ammonium acetate buffers, pH 5.5, were used (Fig. 2). Finally, purification was performed by the Porath column electro-

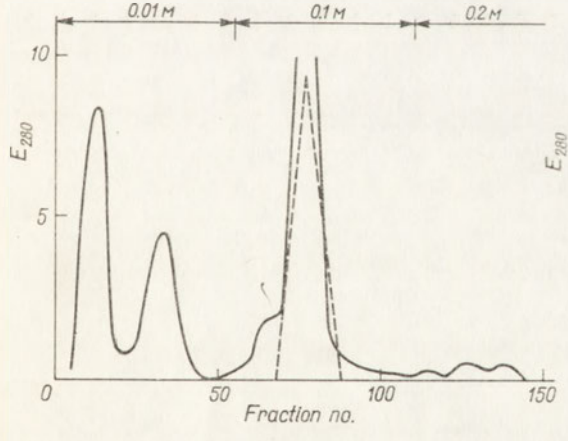

Fig. 1

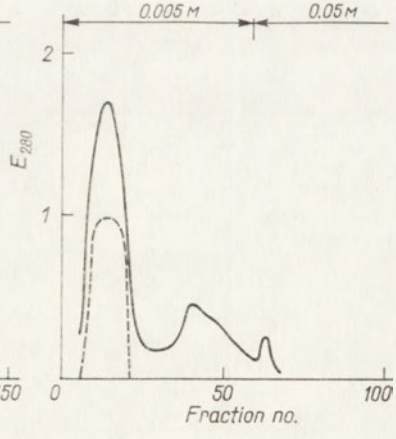

Fig. 2

Fig. 1. Purification of the pituitary inhibitor on DEAE-Sephadex. Details see text. Elution with the indicated concentrations of succinate buffer. ——, Protein; — — —, activity.

Fig. 2. Purification of the pituitary inhibitor on CM-Sephadex. Details see text. Elution with the indicated concentrations of ammonium acetate buffer. ——, Protein; — — —, activity.

phoresis for 16.5 h at 310 V and 10 mA. As adsorbent, Sephadex G-50 in 50 mM-ammonium acetate, pH 5.5, was used; protein was eluted from the column with the same buffer.

The applied purification procedure resulted in about sevenfold purification of the inhibitor, with 8% yield (Table 1). The active fraction examined by high-voltage electrophoresis showed the presence of only one band moving slightly at pH 6.5 towards anode.

Table 1

Purification of the trypsin inhibitor from bovine pituitary

Step	Specific activity (units/mg N)	Total activity (units)	Yield (%)
Initial supernatant	9	73 920	—
Ammonium sulphate 0.45 - 0.90 sat.	41	37 740	51
DEAE-Sephadex	59	28 035	38
CM-Sephadex	64	7 289	10
Column electrophoresis	65.5	6 560	8

Properties of the inhibitor. The bovine pituitary inhibitor was found to contain 14.8% of nitrogen. Its molecular weight determined by means of gel filtration on Sephadex G-100 is 4100 (Fig. 3). EDTA (5×10^{-5} M), mercaptoethanol (5×10^{-8} M) and dithiothreitol (1.5×10^{-3} M) had no effect on the activity of the inhibitor as measured with casein or TAME as trypsin substrates. Preincubation of the inhibitor for 2.5 hours with mercaptoethanol or 30 min with dithiothreitol was also ineffective.

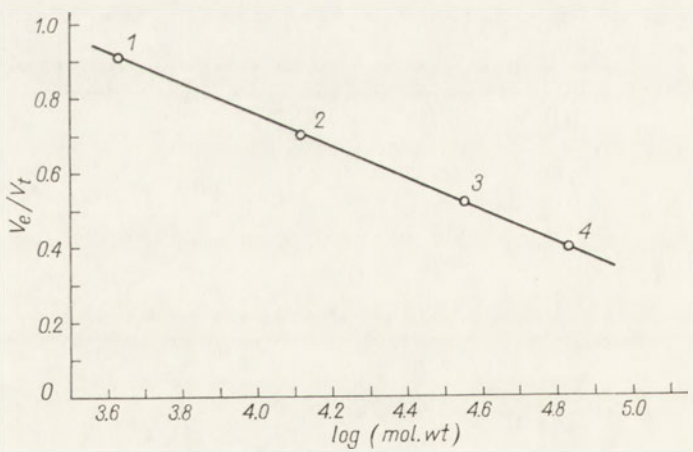

Fig. 3. Determination of molecular weight of the pituitary inhibitor on Sephadex G-100 column in 0.15 M-NaCl. *1*, Inhibitor; *2*, ribonuclease A (mol. wt. 13 000; Rothen, 1940); *3*, pepsin (mol. wt. 35 700; Perlmann, 1955); *4*, bovine serum albumin (mol. wt. 67 000; Loeb & Sheraga, 1956).

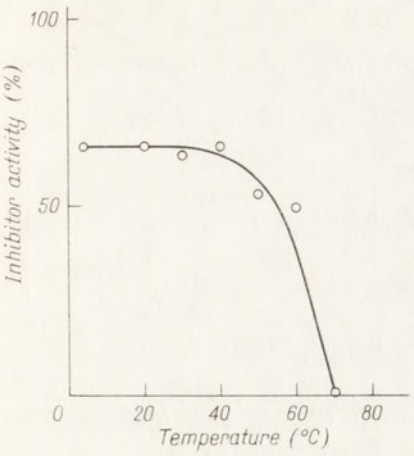

Fig. 4

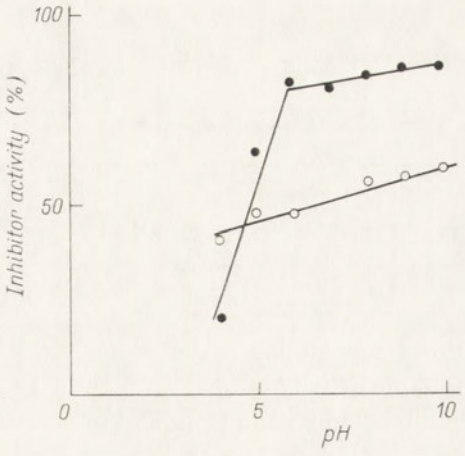

Fig. 5

Fig. 4. Effect of temperature on the activity of the pituitary inhibitor. For details see text.

Fig. 5. Effect of pH on the activity of the pituitary inhibitor. ●, The activity of the inhibitor was measured at different pH values referring to the trypsin activity at corresponding pH values. ○, The inhibitor (0.5% solution) was preincubated for 90 min at room temperature in universal Davies buffers (Rauen, 1964) of different pH values and the activity was measured under standard conditions.

The effect of temperature on the inhibitor activity is shown in Fig. 4. Incubation of the inhibitor without the enzyme for 30 min at 70°C caused its complete inactivation.

Preincubation of the inhibitor with trypsin increased inhibition of the enzyme by only 13%, as measured with casein as substrate, whereas the inhibition measured with synthetic substrate (TAME) could not be obtained without preincubation (Table 2).

Table 2

Effect of preincubation of the pituitary trypsin inhibitor with the enzyme

Determination of activity in pH-stat.

Substrate	Preincubation	Inhibition of trypsin (%)
Casein	—	33
Casein	+	46
TAME	—	0
TAME	+	57

Inhibition of trypsin by the inhibitor at pH below 6 was markedly decreased whereas at pH between 6 and 10 practically no change was observed. Preincubation of the inhibitor at pH 4 - 10 had no significant effect on the inhibitory activity measured under standard conditions (Fig. 5). The extent of inhibition was decreased at higher ionic strength, as shown in Table 3.

Table 3

Effect of ionic strength on the activity of the pituitary trypsin inhibitor

Borate buffer, pH 8.0	Inhibition of trypsin (%)
0.1 M	100
0.1 M+0.3 M-NaCl	100
0.1 M+0.6 M-NaCl	60
0.1 M+0.9 M-NaCl	50

For determination of the molar ratio in binding of the pituitary inhibitor to trypsin, the activity of the enzyme was determined as a function of weight ratio of the inhibitor to trypsin (Fig. 6). For comparison, the results with soyabean trypsin

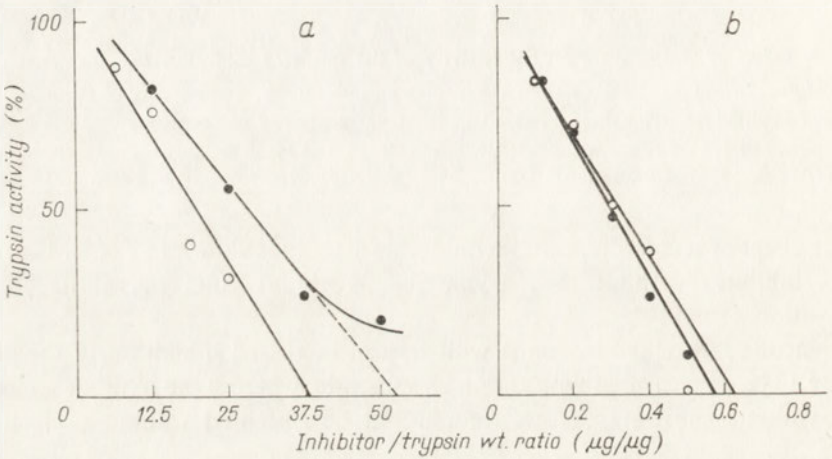

Fig. 6. Inhibition of trypsin activity as a function of the inhibitor/trypsin weight ratio. *a*, Pituitary inhibitor with ○, casein and ●, TAME as substrate; *b*, soyabean trypsin inhibitor with ○, casein and ●, TAME as substrates.

inhibitor are also presented. Assuming that the soyabean trypsin inhibitor reacts with trypsin at 1 : 1 molar ratio (Laskowski & Laskowski, 1954) and that molecular weight of trypsin is 23 300 (Walsh, Kauffman, Sampath Kumar & Neurath, 1964), and of the soyabean trypsin inhibitor 21 500 (Wu & Sheraga, 1962), it has been calculated that the available commercial trypsin preparation contains 62% of the active enzyme. The estimated weight ratio of the pituitary inhibitor to trypsin (Fig. 6) was 53 : 1 with the synthetic substrate, thus the molar ratio of inhibitor (mol. wt. 4100) to trypsin (mol. wt. 23 300) was 471 : 1. In a similar experiment with casein as substrate, the corresponding weight ratio was 38 : 1 and molar ratio 210 : 1.

The bovine pituitary inhibitor (0.25% solution) did not show any inhibitory activity towards chymotrypsin (37.5 μg/ml), pronase (25 μg/ml), papain (0.8 μl/ml) in borate buffer, pH 8.0, pepsin (25 μg/ml) in 0.06 M-HCl, and towards dialysed homogenate of bovine pituitary (in 0.1 M-acetate buffer, pH 6.0).

DISCUSSION

For purification of the inhibitor from the posterior lobe of bovine pituitary a method usually used for purification of proteolytic inhibitors of high molecular weight was chosen. Neither the attempts to precipitate contaminating protein with trichloroacetic acid or perchloric acid were successful, nor purification of the inhibitor by means of insoluble trypsin was satisfactory. The applied fractionation with ammonium sulphate followed by DEAE- and CM-Sephadex chromatography and electrophoresis resulted in isolation and about sevenfold purification of the inhibitor. During purification the chromatography elution diagrams were symmetrical and the final preparation showed on high-voltage electrophoresis at pH 6.5 only one band moving towards anode. This indicates high purity of the obtained preparation.

The isolated inhibitor is a polypeptide of mol. wt. 4100, i.e. it has the lowest molecular weight out of the so far known trypsin inhibitors. The behaviour of the inhibitor on ion-exchange chromatography and high-voltage electrophoresis implies that its isoelectric point is below pH 5.5. The lack of response to mercaptoethanol and dithiothreitol showed that S—S bonds are not important for the inhibitory activity of the inhibitor, nor are metal ions required for its activity. The inhibitor is stable up to 60°C but is completely inactivated at 70°C. Its activity is practically the same at pH 6 - 10.

In the presence of synthetic substrate, the activity of the inhibitor is observed only after preincubation with trypsin. Since synthetic substrate has higher affinity to trypsin than inhibitor (Greene, 1953), the latter cannot bind with trypsin or replace the substrate, although it can be replaced by this substrate in the complex with trypsin. The affinity of protein substrate, e.g. casein, to trypsin is similar to that of the inhibitor and therefore preincubation of the inhibitor with trypsin has a much smaller effect than in the case of synthetic substrate. As the activity of the inhibitor is lower at higher ionic strength, the ionic interaction between inhibitor and trypsin may be suggested.

The molar ratio of the inhibitor to trypsin, 210 : 1 with casein and 471 : 1 with TAME, differs widely from the 1 : 1 ratio found with the other known inhibitors. This could be due to a high dissociation constant of the pituitary inhibitor-trypsin complex, which explains also the deflection from linearity in the graph: activity *versus* inhibitor/trypsin weight ratio (Fig. 6). The high dissociation constant implies weak interaction between the inhibitor and trypsin. This is in accordance with the observed effect of ionic strength on the inhibitor activity. The difference in the inhibitor/trypsin molar ratio found with protein and synthetic substrates is in agreement with different affinities of the inhibitor and the substrates to trypsin. To obtain the same inhibitory effect with the synthetic substrate, a higher molar excess of the inhibitor is necessary. The high inhibitor/trypsin molar ratio can explain the failure in purification of the pituitary inhibitor by means of insoluble trypsin since, due to the high dissociation constant, the inhibitor could not bind to trypsin under these conditions.

Considering inhibitors of proteolytic enzymes as special type substrates (Finkenstadt & Laskowski, 1965), the pituitary inhibitor could be regarded as intermediary between normal substrates decomposed with a high rate constant, and typical inhibitors. The typical inhibitor, e.g. soyabean trypsin inhibitor, does not dissociate from the enzyme under normal conditions although its linkages of the type Arg-X, Lys-X are split by trypsin. Dissociation of the pituitary inhibitor from the inhibitor-trypsin complex is not an effect of the temporary inhibition.

The pituitary inhibitor is highly specific for trypsin. It did not inhibit chymotrypsin, pepsin, papain or pronase. The physiological role of this inhibitor is at present difficult to explain, the more so that it did not lower the proteolytic activity of the pituitary homogenate despite parallel pH optimum of both activities.

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INHIBITOR TRYPSYNOWY Z PRZYSADKI WOŁU

Streszczenie

Przedstawiono metodę izolowania inhibitora trypsynowego z tylnego płata przysadki wołu. Inhibitor oczyszczano stosując frakcjonowanie siarczanem amonu, chromatografię na DEAE- i CM-Sephadex, oraz elektroforezę kolumnową. Inhibitor wykazuje wyłączną specyficzność wobec trypsyny i jest stabilny w temperaturze do 60°C oraz w pH 4 - 10. Optimum aktywności leży w zakresie pH 6 - 10. Inhibitor reaguje z trypsyną w stosunku molowym 210 : 1 w obecności substratu naturalnego i 471 : 1 w obecności estru metylowego *p*-tozylo-L-argininy (TAME).

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PARTIAL PURIFICATION AND PROPERTIES OF THE "CYTOPLASMIC 3-HYDROXYBUTYRATE DEHYDROGENASE" FROM SHEEP KIDNEY

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1. A 35-fold purification of the "cytoplasmic 3-hydroxybutyrate dehydrogenase" from sheep kidney is described. 2. The preparation is active with DL-3-hydroxybutyrate but not with the D(—)-isomer. It also exhibits a relatively high activity with D-gluconate, and the activity ratio with DL-hydroxybutyrate and D-gluconate remains constant during purification. 3. The enzyme absolutely requires thiol-containing reagents (e.g. mercaptoethanol) for both activity and stability. Other properties, as pH dependence and K_m values, are also described. 4. Application of the partially purified enzyme preparation for quantitative determination of acetoacetate is proposed.

Koundakjian & Snoswell (1970) described a soluble 3-hydroxybutyrate dehydrogenase present in the cytoplasmic fraction from kidney and liver of the sheep and regarded this enzyme as the D(—)-3-hydroxybutyrate : NAD oxidoreductase (EC 1.1.1.30). However, Williamson & Kuenzel (1971) have recently shown that sheep kidney cytoplasmic fraction catalyses oxidation of DL-gluconate and L(+)-3-hydroxybutyrate but not D(—)-3-hydroxybutyrate and have therefore postulated that the enzyme described by Koundakjian & Snoswell is, most likely, identical with L-3-hydroxy acid dehydrogenase (EC 1.1.1.45) described by Smiley & Ashwell (1961). Williamson & Kuenzel (1971) purified the enzyme from sheep kidney two-fold and found that the activity ratio with DL-3-hydroxybutyrate, L-3-hydroxybutyrate and DL-gluconate remains essentially unchanged after such purification. They also determined K_m values of the enzyme for 3-hydroxybutyrate and acetoacetate.

The present paper describes a 35-fold purification of the "cytoplasmic 3-hydroxybutyrate dehydrogenase" from sheep kidney cortex and presents further evidence for the identity of this enzyme with L-3-hydroxy acid dehydrogenase. Some properties of the enzyme are also described and the application of the partially purified preparation in enzymic determination of acetoacetate is presented.

MATERIALS AND METHODS

Biological material. Sheep kidneys were obtained from the slaughterhouse. After dissection from the slaughtered animals, they were placed on ice and transported to the laboratory.

Enzyme assays and units. Assays of enzyme activity were carried out by measuring the rate of reduction of NAD^+ or oxidation of NADH at 340 nm. All measurements were made at room temperature (20° - 25°C). The enzyme unit was defined as the amount of enzyme which metabolized one micromol of substrate per one minute under experimental conditions.

Unless otherwise indicated, the assay system for 3-hydroxybutyrate dehydrogenase activity was as follows. In the forward direction:

$$\text{3-hydroxybutyrate} + NAD^+ \rightarrow \text{acetoacetate} + NADH + H^+ \quad (1)$$

33 mM-tris-HCl (pH 8.5), 20 mM-2-mercaptoethanol, 1.5 mM-NAD^+ and 15 mM-DL-3-hydroxybutyrate; in the reverse direction:

$$\text{acetoacetate} + NADH + H^+ \rightarrow \text{3-hydroxybutyrate} + NAD^+ \quad (2)$$

33 mM-phosphate buffer (pH 7.0), 20 mM-2-mercaptoethanol, 0.25 mM-NADH and 20 mM-acetoacetate. Total volume was 1.0 ml, light path 10 mm. The values of pH for the forward and reverse directions as used in the assay systems were found experimentally as optimal (Fig. 1).

L-3-Hydroxy acid dehydrogenase was measured in the same assay system as 3-hydroxybutyrate dehydrogenase in the forward direction except that DL-3-hydroxybutyrate was substituted by 20 mM-D-gluconate.

NADH oxidase was determined in the same medium as for 3-hydroxybutyrate dehydrogenase in the reverse direction except that acetoacetate was omitted.

For the determination of malate dehydrogenase the medium contained hydrazine buffer (0.4 M-hydrazine - 1 M-glycine, pH 9.5), 1.5 mM-NAD^+ and 15 mM-L-malate (Hohorst, 1963).

Lactate dehydrogenase was measured in the medium containing 50 mM-phosphate buffer (pH 7.5), 0.14 mM-NADH and 0.31 mM-pyruvate (Bergmeyer, Bernt & Hess, 1963).

Analytical procedures. Protein was determined by the biuret method (Gornall, Bardawill & David, 1949) with bovine serum albumin as standard. Samples containing ammonium sulphate were subjected to dialysis prior to protein determination. Chemical determination of 3-hydroxybutyrate formed during enzymic oxidation of palmitoyl carnitine was carried out as follows: From the sum of ketone bodies, determined by the method of Lester & Greenberg (1948) as modified by Bremer (1966), was subtracted the amount of acetoacetate measured according to Walker (1954).

Chemicals. The chemicals used were as follows: NADH, sodium DL-3-hydroxybutyrate, oxaloacetic acid and dithioerythritol (2,3-dihydroxy-1,4-dithiobutane)

from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); L-malic acid, sodium pyruvate and 2-mercaptoethanol from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., England); NAD^+ from Reanal (Budapest, Hungary); cysteine from Fluka AG (Buchs SG, Switzerland); and D-gluconic acid from Polskie Odczynniki Chemiczne (Gliwice, Poland). Other reagents used were of analytical grade. Acetoacetate was obtained by hydrolysis of its ethyl ester as described by Seeley (1955). DEAE-Sephadex (Pharmacia, Uppsala, Sweden) was pretreated as described by Bergmeyer, Gawehn, Klotzsch, Krebs & Williamson (1967) and calcium phosphate gel was prepared according to Keilin & Hartree (1938) and adjusted to pH 7.4.

EXPERIMENTAL AND RESULTS

Purification of the enzyme

Kidney cortex (400 g wet weight) was cut into small pieces and homogenized in a Teflon-glass homogenizer with 1000 ml of the medium containing 125 mM-KCl, 4 mM-tris-HCl (pH 7.4), 5 mM-2-mercaptoethanol and 0.5 mM-EDTA. The homogenate was centrifuged at 4500 *g* for 15 min and the resulting supernatant again at 100 00 *g* for 60 min (Table 1, step 1). The clear supernatant was adjusted to 0.35 saturation with saturated ammonium sulphate. The precipitate was removed by centrifugation and discarded, and the supernatant was made 0.7 saturated with solid ammonium sulphate (21.9 g per 100 ml). The precipitate was collected, dissolved in 100 ml of 50 mM-phosphate buffer (pH 7.8) containing 10 mM-2-mercaptoethanol and dialysed against a 40-fold volume of 10 mM-phosphate buffer, pH 7.8 (step 2). Any undissolved material was removed and discarded and the clear solution was

Table 1

Purification procedure of the "cytoplasmic 3-hydroxybutyrate dehydrogenase" from sheep kidney

DEAE-Sephadex chromatography was followed in steps 3, 5 and 7 by precipitation of enzymic protein with 0.55 ammonium sulphate sat. and dialysis; the activity values refer to the dialysed preparation.

Step no.	Treatment	Total activity (units)	Protein (mg)	Specific activity (units/mg protein)	Purification (×)	Yield (%)
1 a	Supernatant 4 500 *g*	460	22 660	0.020	1	100
1 b	Supernatant 100 000 *g*	420	16 150	0.026	1.3	91.5
2	Ammonium sulphate 0.35 - 0.7 sat.	395	11 290	0.035	1.7	86
3	DEAE-Sephadex (1st)	215	3 410	0.068	3.4	47
4	Calcium phosphate (1st)	170	1 420	0.120	6.0	37
5	DEAE-Sephadex (2nd)	120	405	0.296	14.8	26
6	Calcium phosphate (2nd)	90	240	0.380	19	19.5
7	DEAE-Sephadex (3rd)	48	68	0.705	35	10

mixed with 5 g of DEAE-Sephadex A-50. After 15 min the mixture was filtered. The filtrate contained about half of the enzyme activity of almost twice as high specific activity as the original solution. The remaining activity could be eluted by 0.2 M-phosphate buffer (pH 7.8), containing increasing concentrations of NaCl. As shown in Table 2, 0.2 M-phosphate buffer alone and the same buffer with 0.1 M-NaCl eluted most of the enzyme adsorbed, of high specific activity. Further increasing the concentration of NaCl resulted in fractions containing little enzyme of low specific activity. Therefore, only the filtrate and the first two eluted fractions were pooled and used for further purification. The enzyme was precipitated by 0.55 saturation of ammonium sulphate (32.6 g solid ammonium sulphate per 100 ml), dissolved in 100 ml of 50 mM-phosphate buffer (pH 7.8) containing 10 mM-mercaptoethanol and dialysed against a 40-fold volume of 10 mM-phosphate buffer for 5 hours (step 3). The enzyme solution was then mixed with calcium phosphate gel, taking 750 mg of the gel per 1 g protein, and the mixture was immediately centrifuged. This treatment removed inert proteins adsorbed on the gel, resulting in an about twofold purification (step 4). The supernatant was treated with DEAE-Sephadex as described before, followed by ammonium sulphate precipitation, dissolving in 50 mM-phosphate buffer (pH 7.8) and dialysis (step 5). This was followed by the second calcium phosphate gel (step 6) and the third DEAE-Sephadex treatments (step 7). The final precipitate was dissolved in 20 ml of 50 mM-phosphate buffer (pH 7.8) containing 20 mM-mercaptoethanol and dialysed for one hour against a 400-fold volume of 10 mM-phosphate buffer (pH 7.8) supplemented with 20 mM-mercaptoethanol. The final solution was stored at -15°C. It contained 10% of the total enzyme activity present in the supernatant after 4 500 *g* centrifugation, the specific activity being 35 times higher (Table 1).

Table 2

Purification of the "cytoplasmic 3-hydroxybutyrate dehydrogenase" by means of DEAE-Sephadex A-50

For experimental details see the text. Fractions no. 1, 2 and 3 were combined and used for further purification. Elution with 0.2 M-phosphate buffer supplemented with the indicated concentrations of NaCl.

No.	Fraction	Volume (ml)	Total activity (units)	Recovery (%)	Specific activity (units/mg protein)
	Enzyme solution from step 2	150	350	(100)	0.031
1	Filtrate after Sephadex treatment	180	180	51	0.052
2	Elution with 0.2 M-phosphate buffer	400	68	19	0.089
3	+0.1 M-NaCl	200	44	13	0.075
4	+0.2 M-NaCl	200	27	8	0.044
5	+0.4 M-NaCl	200	20	6	0.030
6	+0.8 M-NaCl	200	10	3	0.028

All steps of purification were carried out at 0° - 4°C. The entire procedure lasted three working days. During the nights, the enzyme was kept in the dissolved state (never as ammonium sulphate precipitate) frozen at -15°C. For good preservation of the enzyme activity it appeared important to perform all manipulations with the unfrozen enzyme as quickly as possible and to maintain mercaptoethanol present during all steps of the procedure.

Properties of the partially purified preparation

As shown in Table 3, the partially purified preparation of "cytoplasmic 3-hydroxybutyrate dehydrogenase" was slightly contaminated with malate dehydrogenase and lactate dehydrogenase, but contained no NADH oxidase. Its activity in the reverse direction (reaction 2) was, under experimental conditions, approximately 40% that in the forward direction (reaction 1). In agreement with the suggestion of Williamson & Kuenzel (1971) that the "cytoplasmic 3-hydroxybutyrate dehydrogenase" is, in fact, L-3-hydroxy acid dehydrogenase, our purified preparation exhibited a considerably high activity with D-gluconate (Table 3). In order to verify the suggestion of Williamson & Kuenzel (1971) we measured the activity with both DL-3-hydroxybutyrate and D-gluconate at every step of the procedure. This time, the purification was 18-fold in respect to 3-hydroxybutyrate dehydrogenase and 22-fold for gluconate dehydrogenase activity. As shown in Table 4, the ratio between the two activities was similar at all steps of the procedure and oscillated between 0.5 and 0.6.

Table 3

Enzymic activities of the partially purified "cytoplasmic 3-hydroxybutyrate dehydrogenase" from sheep kidney

The assay systems for 3-hydroxybutyrate dehydrogenase activity in the forward and reverse directions, and for the indicated enzymes are described under Materials and Methods. The enzyme preparation contained 2.75 mg protein/ml. Relative activity refers to the activity with DL-3-hydroxybutyrate taken as unity.

Enzymic activity	Substrate	Activity	
		units/ml	relative
3-Hydroxybutyrate dehydrogenase (forward direction)	DL-3-Hydroxybutyrate	1.92	1
3-Hydroxybutyrate dehydrogenase (reverse direction)	Acetoacetate	0.77	0.40
L-3-Hydroxy acid dehydrogenase	D-Gluconate	1.13	0.59
NADH oxidase	NADH	0.00	0.00
Malate dehydrogenase	L-Malate	0.084	0.04
Lactate dehydrogenase	Pyruvate	0.054	0.03

Effect of pH was measured with 3-hydroxybutyrate and acetoacetate as substrates. As shown in Fig. 1, the activity in the forward direction (reaction 1) increased several-fold from pH 7.0 to 8.6 and showed a plateau between pH values 8.6 and 9.0. Contrary to this, the activity in the reverse direction (reaction 2) had a broad optimum between pH 7 and 8.

Apparent Michaelis constants for the partially purified "cytoplasmic 3-hydroxybutyrate dehydrogenase" in respect to oxidized and reduced nicotinamide nucleotides, acetoacetate and hydroxy acids are shown in Table 5. It can be seen that the values for NAD^+ and NADH are low, whereas those for the acids are relatively high. Assuming that the enzyme is active only with the L(+)-isomer of 3-hydroxybutyrate (Williamson & Kuenzel, 1971; and the evidence presented below), the true value of K_m for this substrate appears to be lower. If the D-isomer has no effect on binding of the L-substrate, the K_m value is half of that obtained with the DL-racemate, i.e. 2.5 mM. However, if the D-form inhibits competitively, then the true K_m is even lower.

The stereospecificity of the enzyme was checked with DL-3-hydroxybutyrate and D(—)-3-hydroxybutyrate; since synthetic D-isomer was not available, it was

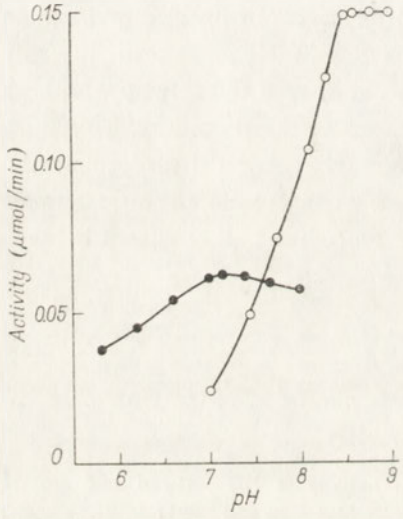

Fig. 1

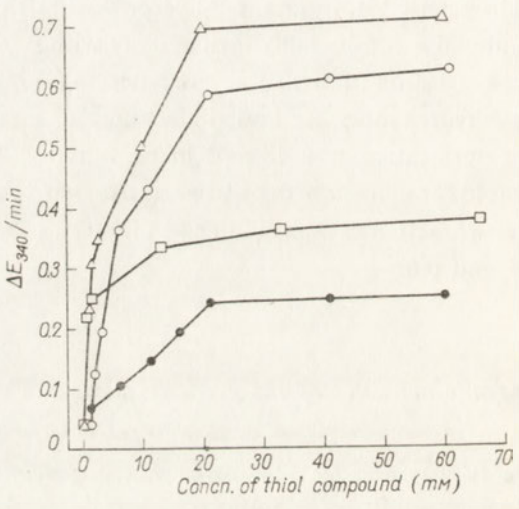

Fig. 2

Fig. 1. Effect of pH on the activity of partially purified "cytoplasmic 3-hydroxybutyrate dehydrogenase". Assay conditions were as described under Materials and Methods, except that pH values were changed. The medium contained 0.21 mg protein of the purified preparation (step 7). ○, Forward direction (DL-3-hydroxybutyrate as substrate, tris-HCl buffer); ●, reverse direction (acetoacetate as substrate, phosphate buffer).

Fig. 2. Effect of thiol compounds on the activity of partially purified "cytoplasmic 3-hydroxybutyrate dehydrogenase". The assay medium contained 0.1 enzyme unit as measured with 3-hydroxybutyrate as substrate. The stock enzyme solution always contained 20 mM-mercaptoethanol, therefore the concentration of this compound in the final assay medium could not be decreased below 1 mM. For the same reason, 1 mM-mercaptoethanol was also present in all samples where other thiol compounds were tested. Open symbols, forward direction (with DL-3-hydroxybutyrate) with mercaptoethanol (○), dithioerythritol (△), and cysteine (□); full symbols, reverse direction (with acetoacetate as substrate) with mercaptoethanol (●).

Table 4

Activity of the "cytoplasmic 3-hydroxybutyrate dehydrogenase" with DL-*3-hydroxybutyrate and* D-*gluconate at successive steps of purification*

Purification steps are described in Table 1 and in the corresponding text; enzyme activity was measured as described under Materials and Methods.

Step no.	Activity with DL-3-hydroxybutyrate		Activity with D-gluconate		Ratio [B]/[A]
	total (units) [A]	specific (units/mg protein)	total (units) [B]	specific (units/mg protein)	
1 a	150	0.008	72	0.004	0.48
1 b	140	0.011	70	0.005	0.50
2	135	0.015	73	0.008	0.54
3	100	0.050	58	0.029	0.58
4	60	0.088	34	0.052	0.57
5	38	0.110	23	0.066	0.60
7	16	0.145*	9.5	0.086**	0.59

* Purification 18×; ** purification 22×.

Table 5

Apparent Michaelis constants of the "cytoplasmic 3-hydroxybutyrate dehydrogenase" from sheep kidney

Enzyme activity was measured as described under Materials and Methods

Substrate	K_m (mM)
NAD^+	0.2
NADH	0.05
DL-3-Hydroxybutyrate	5
Acetoacetate	7
D-Gluconate	10

obtained enzymically using rat liver mitochondria which oxidized palmitoyl carnitine in a system described previously (Wojtczak, 1968). Portions of the incubation mixture were inactivated by perchloric acid, neutralized with $KHCO_3$ and assayed with the enzyme preparation. No activity, as measured by the reduction of NAD^+, could be detected although the portions contained substantial amounts of 3-hydroxybutyrate as determined chemically. Synthetic DL-3-hydroxybutyrate used in the same amounts produced a measurable enzymic reaction.

Dependence on thiol groups. The partially purified preparation of "cytoplasmic 3-hydroxybutyrate dehydrogenase" from sheep kidney exhibits an absolute requirement for thiol compounds for its activity. As shown in Fig. 2, optimum activity could be obtained in the presence of mercaptoethanol or dithioerythritol at concentrations of 20 mM or above. Cysteine was also effective, although to a smaller extent. The presence of thiol group-protecting reagents was necessary for full activity

in both directions of the reaction. Lowering of mercaptoethanol concentration below 1 mM resulted in partial inactivation of the enzyme. Subsequent increase in concentration of mercaptoethanol to 20 mM partially restored the activity. Therefore, care should be taken not to expose the enzyme to solutions devoid of thiol compounds.

Stability. The enzyme in the partially purified form is very stable when stored frozen at -15°C in the presence of 20 mM-mercaptoethanol. Under these conditions no detectable change of activity could be observed after 5 months.

Application of the "cytoplasmic 3-hydroxybutyrate dehydrogenase" for enzymic determination of L(+)*-3-hydroxybutyrate and acetoacetate*

Because of the high K_m values for acetoacetate and 3-hydroxybutyrate the enzyme cannot be used for the quantitative determination of these substances in a way analogous to that applied with bacterial D(—)-3-hydroxybutyrate dehydrogenase (Williamson, Mellanby & Krebs, 1962), i.e. by allowing the reaction to go to completion and measuring the resulting reduction or oxidation of nicotinamide nucleotides. However, with the sheep enzyme, advantage can be taken of the fact that the initial reaction rate is proportional to acetoacetate at concentrations below 1 mM, i.e. at concentrations usually found in biochemical assays (cf. e.g. Wojtczak, 1968). Extinction change due to reduction of acetoacetate was usually measured during the first 5 min of the reaction. Using known concentrations of acetoacetate to calibrate the enzyme preparation used (Fig. 3), one can determine the amount of acetoacetate in the assayed material. As the enzyme is active only with the "non-physiological" L-isomer of 3-hydroxybutyrate, its application for determination of 3-hydroxybutyrate has a limited importance in biochemical studies.

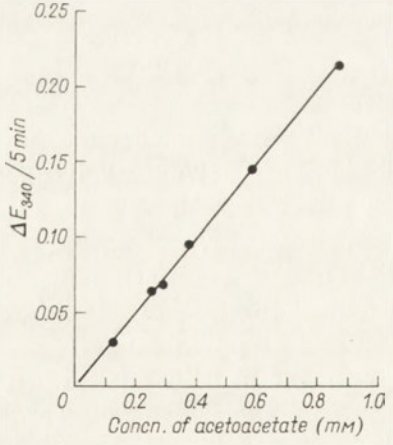

Fig. 3. Effect of concentration of acetoacetate on the reaction rate. Assay conditions were as described under Materials and Methods, except that the concentration of NADH was increased to 0.30 mM. The medium contained 0.04 enzyme unit. The reaction was started by the addition of NADH, and extinction changes after the first 5 min are plotted. The assay volume was 1.0 ml.

Table 6

Interference with determination of acetoacetate by the "cytoplasmic 3-hydroxybutyrate dehydrogenase"

The assay medium contained 0.465 mM-acetoacetate and interfering substances at indicated concentrations. Other conditions were as described in the legend to Fig. 3.

Interfering substance	Concentration (mM)	ΔE_{340} after 5 min	Error (%)
None		—0.110	—
DL-3-Hydroxybutyrate	0.46	—0.110	0
	0.92	—0.110	0
	1.8	—0.110	0
	4.6	—0.090	—18
L-Malate	0.46	—0.110	0
	1.5	—0.095	—14
	4.5	—0.065	—41
Oxaloacetate	0.15	—0.185	+68
	0.30	—0.125	+14
	0.46	—0.095	—14
	0.92	—0.100	—9
Pyruvate	0.1 - 4.6	—0.110	0

The interference by DL-3-hydroxybutyrate, malate, oxaloacetate and pyruvate is shown in Table 6. It is seen that DL-3-hydroxybutyrate at concentrations up to four times higher than that of acetoacetate does not interfere with acetoacetate determination and the interference by hydroxybutyrate concentrations 10 times higher does not exceed 20%. L-Malate is without effect only at a low concentration equal to that of acetoacetate. At higher concentrations, it exhibits a negative interference (lowering of the reaction rate), apparently due to contamination of the enzyme preparation with malate dehydrogenase. This contamination is also responsible for the positive interference (increasing of the reaction rate) by small concentrations of oxaloacetate. Negative interference by higher concentrations of oxaloacetate is more difficult to explain. It might be due to inhibition of the enzyme by oxaloacetate.

DISCUSSION

The present investigation provides further support to the suggestion (Williamson & Kuenzel, 1971) that "cytoplasmic 3-hydroxybutyrate dehydrogenase" from sheep kidney (Koundakjian & Snoswell, 1970) is identical with L-3-hydroxy acid dehydrogenase described earlier by Smiley & Ashwell (1961). The parallel purification of the activities with 3-hydroxybutyrate and D-gluconate and high activity of the partially purified preparation with both substances are good evidence. The ratio of the activities with 3-hydroxybutyrate and D-gluconate is in our preparation almost

identical with that found by Williamson & Kuenzel (1971) for the sheep enzyme but differs from the corresponding value for hog kidney enzyme (Smiley & Ashwell, 1961).

We succeeded in an up to 35-fold purification of the enzyme, in contrast to a twofold purification described by Williamson & Kuenzel (1971). This was most likely due to the protective effect of mercaptoethanol used at all steps of our purification procedure. A highly purified L-3-hydroxy acid dehydrogenase has been recently obtained from insect material (Borack & Sofer, 1971).

K_m values for acetoacetate and L-3-hydroxybutyrate obtained in this investigation are about three times lower than those reported by Williamson & Kuenzel (1971). The reason for this discrepancy is not known. The relatively high K_m values, in the range of millimolarity, for acetoacetate and 3-hydroxyacids tested (Table 5) make it doubtful that these substances are natural substrates for the enzyme. The biological role of the enzyme remains, therefore, not fully understood.

Our partially purified preparation of L-hydroxy acid dehydrogenase ("cytoplasmic 3-hydroxybutyrate dehydrogenase") differs somewhat in its properties, namely the activity ratio with various substrates, pH dependence and K_m values, from the enzyme from hog kidney studied by Smiley & Ashwell (1961). The most important difference consists, however, in the sensitivity to thiol-containing compounds. The presence of mercaptoethanol or other thiol compounds was absolutely required for both the stability of the sheep kidney enzyme and its activity in both directions, whereas the enzyme from hog kidney was stimulated only by cysteine, and exclusively in the forward direction (Smiley & Ashwell, 1961). Moreover, the presence of mercaptoethanol was needed at all steps of our purification procedure whereas Smiley & Ashwell (1961) succeeded in a 100-fold purification of the hog enzyme without such treatment. This indicates different stability of SH groups in both enzymes.

The present study shows, in addition, that the partially purified "cytoplasmic 3-hydroxybutyrate dehydrogenase" from sheep kidney can be used for the quantitative determination of acetoacetate in biological material and biochemical experiments. The enzymic assay for acetoacetate is used when substances interfering in the chemical determination (Walker, 1954), e.g. malonate, are present. In contrast to bacterial D-3-hydroxybutyrate dehydrogenase the rate of the reaction rather than the stoichiometric oxidation of NADH is used as the measure of the amount of acetoacetate, which presents a certain disadvantage.

Some limitation in the use of the enzymic method comes from the interference by carboxylic acids (Table 6). This interference has been, however, observed also in the case of the bacterial D(—)-3-hydroxybutyrate dehydrogenase (Williamson *et al.*, 1962). On the other hand, relative simplicity of the purification procedure of the sheep enzyme, the availability of the starting material and satisfactory stability of the purified enzyme preparation are the factors which make the new procedure worthy of consideration.

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CZĘŚCIOWE OCZYSZCZENIE I WŁAŚCIWOŚCI "CYTOPLAZMATYCZNEJ DEHYDROGENAZY 3-HYDROKSYMAŚLANU" Z NERKI OWCY

Streszczenie

1. Opisano procedurę oczyszczania "cytoplazmatycznej dehydrogenazy 3-hydroksymaślanu" z nerki owcy; uzyskano 35-krotny wzrost aktywności właściwej.

2. Enzym jest aktywny wobec DL-3-hydroksymaślanu lecz nieaktywny wobec izomeru D(—). Wykazuje także znaczną aktywność wobec D-glukonianu, przy czym stosunek aktywności wobec DL-hydroksymaślanu i D-glukonianu pozostaje niezmieniony w czasie oczyszczania.

3. Enzym wymaga związków tiolowych zarówno dla swej aktywności, jak i trwałości. Zbadano także inne właściwości enzymu, jak zależność od pH oraz stałe K_m.

4. Opracowano metodę ilościowego oznaczania acetooctanu opartą na zastosowaniu częściowo oczyszczonego preparatu dehydrogenazy.

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PURINE METABOLISM IN WHEAT SHOOTS TREATED WITH AMINOTRIAZOLE

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Aminotriazole does not affect biosynthesis of adenine, as measured by incorporation of [U-^{14}C]glycine into the free-purine fraction of wheat shoots. It inhibits by 20% incorporation of [U-^{14}C]glycine and [8-^{14}C]adenine into the polynucleotide fraction; this effect is not observed in the shoots grown in dark.

Aminotriazole was reported to interfere with purine biosynthesis in various organisms, and adenine partially reversed the inhibitory effect of this compound on growth of bacteria (Hilton, Kearney & Ames, 1965), yeast (Hilton, 1960; Kłopotowski & Bagdasarian, 1966), algae (Wolf, 1962; Boney, 1963) and higher plants (Bartels & Wolf, 1965).

Two mechanisms of the interference by aminotriazole have been postulated, namely the effect on purine biosynthesis (Hilton *et al.*, 1965), and on the polymerization process leading to polynucleotides (Hilton, 1969).

In the present work, we have attempted to get some information on the mechanism of aminotriazole action in higher plants.

METHODS

Plant. Wheat (*Triticum vulgare*), variety "Dańkowska 40", was used.

Conditions of growth and incorporation of labelled compounds. Wheat seeds were placed on folded gauze soaked with tap water and germinated in large dishes for seven days at room temperature. Except for one experiment, the plants were grown in natural daylight. The shoots were cut off and incubated at room temperature, for up to 4 h, in aqueous solutions with [U-^{14}C]glycine or [8-^{14}C]adenine, supplemented with aminotriazole and/or L-histidine.

Extraction, purification and assays of purine compounds. Frozen plants were ground in cooled mortar and 10 g samples of the powder were extracted successively with 10 ml of 0.6 M-perchloric acid and five times with 35 ml portions of 0.3 M-perchloric

acid, centrifugation following each extraction. Separation and analysis of purines was carried out essentially according to Rybicka, Buchowicz & Reifer (1967); hydrolysis of purine compounds was performed with 1 N-HCl for 1 h at 100°C. The purines adsorbed on Dowex 1X 8 (chloride form) were eluted with water and analysed by paper chromatography on Whatman no. 1 filter paper, using *n*-butanol saturated with water and conditioned with ammonia, and Wyatt's (1955) solvent mixture (340 ml of isopropanol, 82 ml of concentrated HCl and water up to 500 ml).

The solid residue after perchloric acid extractions was suspended in 1 N-HCl and polynucleotides were hydrolysed at 100°C for 1 h. Further procedure was the same as used for the acid-soluble fraction except that Dowex chromatography was omitted.

Chemicals. Aminotriazole was a product of Fluka GmbH (Buchs, Switzerland); [U-^{14}C]glycine (sp. act. 113 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.), and [8-^{14}C]adenine (sp. act. 35 mCi/mmol) from the Radiochemical Centre (Amersham, England).

RESULTS AND DISCUSSION

The data given in Table 1 demonstrate that incorporation of [U-^{14}C]glycine into adenine and guanine of the acid-soluble fraction extracted from wheat shoots was not affected by aminotriazole. The total amount of [U-^{14}C]glycine taken up was practically the same in control and aminotriazole-treated shoots, 6.9×10^5 and 6.4×10^5 c.p.m. or 67% and 62%, respectively.

Table 1

Incorporation of [U-^{14}C]*glycine into purine bases in cut-off wheat shoots*

Ten gram samples of cut-off wheat shoots were incubated for 1 h at 20°C with 0.042 µmol of [U-^{14}C]glycine (sp. act. 2.46×10^7 c.p.m./µmol) and 100 µmol of aminotriazole. Average values from two separate experiments, each run in duplicate, are given.

Incubation mixture	Acid-soluble fraction		Acid-insoluble fraction	
	Adenine	Guanine	Adenine	Guanine
	c.p.m./µmol		c.p.m./µmol	
Glycine	1800	190	17	<5
Glycine+aminotriazole	1900	190	13	<5

If diffusion of aminotriazole parallelled that of glycine, the effective concentration of the herbicide within the shoots should be about 10 mM. A concentration twice as high was needed to produce in *S. typhimurium* a growth-inhibiting shortage of purine compounds (Hilton *et al.*, 1965), but 5 and 10 mM-aminotriazole was almost equally effective in inhibiting aminoimidazole production (Hulanicka, Kłopotowski & Bagdasarian, 1969).

In contrast to radioactivity of free-purine fraction, the specific activity of adenine in acid-insoluble fraction was lower by about 20% in aminotriazole-treated shoots, irrespective whether [U-^{14}C]glycine or [8-^{14}C]adenine was used as a polynucleotide adenylic acid precursor (Tables 1, 2). Incorporation of radioactive adenine into polynucleotide fraction was followed as a function of time in two separate experiments. Each time the extent of inhibition was the same. Radioactivity of guanine was very low and therefore determinations were subject to considerable error.

A similar effect of aminotriazole on polynucleotide biosynthesis has been observed with yeast (Hilton & Kearney, 1965) and bacteria (Hilton, 1969). However, inhibition of incorporation of labelled adenine could be reversed by histidine, and

Table 2

Incorporation of [8-^{14}C]*adenine into polynucleotide fraction in cut-off wheat shoots grown in light*

Samples of cut-off wheat shoots, 2.5 g, were incubated for 1, 2 and 4 h at 20°C with 0.015 or 0.007 µmol of [8-^{14}C]adenine (sp. act. 7.6×10^6 c.p.m./µmol) in experiments 1 and 2 respectively. The incubation solutions were supplemented with 25 µmol of aminotriazole or/and 4 µmol of L-histidine. Each value is an average from two separately incubated samples, percent inhibition being given in parentheses.

Incubation mixture	Sp. act. (c.p.m./µmol)		
	1 h	2 h	4 h
Experiment 1			
Adenine	412	691	1150
Adenine+aminotriazole	273 (33)	530 (23)	833 (28)
Experiment 2			
Adenine	—	468	632
Adenine+aminotriazole	—	385 (18)	546 (14)
Adenine+L-histidine	—	380	632
Adenine+aminotriazole+L-histidine	—	345 (9)	495 (22)

Table 3

Incorporation of [8-^{14}C]*adenine into adenine of nucleotide fraction by wheat shoots grown in dark*

Samples of wheat shoots, 2.5 g, were incubated for 4 h at 20°C with 0.007 µmol of [8-^{14}C]adenine (sp. act. 7.6×10^6 c.p.m./µmol) and 25 µmol of aminotriazole. Each value is an average from two separately incubated samples.

Incubation mixture	Sp. act. (c.p.m./µmol)
Experiment 1	
Adenine	710
Adenine+aminotriazole	700
Experiment 2	
Adenine	574
Adenine+aminotriazole	660

therefore it could be a secondary phenomenon. In our experiments, histidine had no effect (Table 2).

The low inhibition of nucleic acid synthesis in wheat shoots by aminotriazole, observed in our experiments, suggests that the herbicide could inhibit the biosynthesis of a specific fraction of cellular nucleic acids. Bartels, Matsuda, Siegel & Weier (1967) have shown that aminotriazole affects only chloroplast ribosomes. Therefore, the incorporation of adenine into polynucleotide fraction was repeated with wheat shoots grown in dark. Under these conditions, presumably only the latent form of chloroplasts exists. Table 3 shows that aminotriazole was without a significant effect on specific radioactivity of adenine in the polynucleotide fraction.

Further investigations are needed to ascertain whether aminotriazole is a weak inhibitor of biosynthesis of a large fraction of polynucleotides, or a strong inhibitor of a quantitatively minor fraction.

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METABOLIZM PURYN W KIEŁKACH PSZENICY PODDANYCH DZIAŁANIU AMINOTRIAZOLU

Streszczenie

Aminotriazol nie wpływa na włączanie [U-^{14}C]glicyny we frakcję purynową w uciętych kiełkach pszenicy. Hamuje on w 20% włączanie [U-^{14}C]glicyny i [8-^{14}C]adeniny we frakcję polinukleotydową kiełków rosnących na świetle, lecz nie hamuje włączania obu prekursorów w kiełkach rosnących w ciemności.

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THE STRUCTURE OF POLY 2′-*O*-METHYLCYTIDYLIC ACID AND ITS COMPLEXES WITH POLYINOSINIC ACID

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1. In neutral and alkaline media poly 2′-*O*-methylcytidylic acid (poly 2′-*O*-MeC) exists in the form of a single strand, with properties similar to those of poly rC, and not poly dC.

2. In acid medium, poly 2′-*O*-MeC forms a twin-stranded helix, the properties of which are again similar to those of poly rC, and markedly different from those of the acid twin-helical form of poly dC.

3. Poly 2′-*O*-MeC forms a double-stranded helical complex with poly rI. The T_m for the helix-coil transition of this complex is intermediate between those for the corresponding complexes, with poly rI, of poly rC and poly dC.

4. The conformational properties of the double-stranded poly rI : 2′-*O*-MeC as a function of ionic strength have been examined.

5. Poly 2′-*O*-MeC does not complex with poly dI under experimentally available conditions of temperature and ionic strength.

6. The similarity in physico-chemical properties of poly 2′-*O*-MeC to poly rC is discussed, in part in relation to the contrasting differences in known biological activity.

The synthesis, and studies of the properties, of a series of polyribonucleotides in which the 2′-hydroxyls are replaced by 2′-*O*-methyl (Rottman & Henlein, 1968; Żmudzka, Janion & Shugar, 1969c; Żmudzka & Shugar, 1970) were carried out primarily with a view to elucidating the role of the 2′-hydroxyl in the structure and biological function of nucleic acids. The contrasting properties of DNA and RNA, due to the presence in the latter of the 2′-hydroxyl, are reflected in the pronounced differences in physico-chemical properties between synthetic polynucleotides containing ribose or deoxyribose. These differences were for some time ascribed to the presence, in the polyribonucleotides, of a supplementary hydrogen bond in which

the 2′-OH[1] served as a donor, and a pyrimidine $C_{(2)}=O$, a purine ring $N_{(3)}$, or a phosphate oxygen (in the same residue) as acceptor (Ts'o, Rapaport & Bollum, 1966; Brahms, Maurizot & Michelson, 1967; see, however, Rabczenko & Shugar, 1971).

Although this proposal was not based on any direct experimental evidence and, indeed, was in disagreement with X-ray diffraction data (see Rabczenko & Shugar, 1971, for more detailed discussion), it proved surprising to find that poly 2′-*O*-MeA (Bobst, Rottman & Cerutti, 1969), poly 2′-*O*-MeC (Żmudzka *et al.*, 1969c) and poly 2′-*O*-MeU (Żmudzka & Shugar, 1970, 1971), all three of which are incapable of forming such a hydrogen bond, nonetheless mimic the physico-chemical properties of the corresponding poly*ribo*nucleotides and, like the latter, differ in a number of respects from the corresponding polydeoxyribonucleotides. While this does not necessarily constitute conclusive evidence for the presence or absence of a 2′-OH hydrogen bond in the natural polyribonucleotides, it at least demonstrates that such hydrogen bonding is not a prerequisite for the formation of helical structures of the latter.

The present paper presents a detailed account of some of the properties of poly 2′-*O*-MeC, particularly from the point of view of the striking differences known to exist between the acid and neutral forms of poly rC on the one hand, and poly dC on the other (Inman, 1964; cf. Żmudzka, Bollum & Shugar, 1969a). Furthermore, since it was already known from preliminary observations (Żmudzka *et al.*, 1969c) that poly 2′-*O*-MeC forms a double-stranded helix with poly rI, it appeared desirable to examine in greater detail the ability of poly 2′-*O*-MeC to complex with poly rI and poly dI. It was also expected that such complexes might prove useful as interferon inducers (Field, Tytell, Lampson & Hilleman, 1967), the more so in that poly 2′-*O*-MeC is relatively resistant to various nucleases and, as might be anticipated, is completely resistant to alkaline ribonuclease (Żmudzka *et al.*, 1969c), since the absence of the ribose 2′-hydroxyl eliminates any possibility of transphosphorylation.

MATERIALS AND METHODS

Poly 2′-*O*-MeC, obtained by polymerization of 2′-*O*-MeCDP with *Micrococcus lysodeikticus* polynucleotide phosphorylase (Matthaei *et al.*, 1967) under conditions elsewhere described (Janion, Żmudzka & Shugar, 1970), exhibited an ε_{267} of

[1] The following abbreviations are used in this text: 2′-OH, ribose 2′-hydroxyl; 2′-*O*-MeC, 2′-*O*-methylcytidine; 2′-*O*-MeU, 2′-*O*-methyluridine; 2′-*O*-MeA, 2′-*O*-methyladenosine; 2′-*O*-MeCDP, 2′-*O*-methylcytidine-5′-pyrophosphate, and similarly for 2′-*O*-MeUDP and 2′-*O*-MeADP; 2′-*O*-MeCTP, 2′-*O*-methylcytidine-5′-triphosphate, and similarly for 2′-*O*-MeUTP and 2′-*O*-MeATP; poly rC, polyribocytidylic acid; poly 5MerC, poly 5-methylcytidylic acid; poly rU, polyribouridylic acid; poly rT, polyribothymidylic acid; poly rI, polyriboinosinic acid; poly rX, polyriboxanthylic acid; poly dC, poly dU, poly dI, etc. are the corresponding deoxy polymers. Poly 2′-*O*-MeC, poly 2′-*O*-methylcytidylic acid; poly 2′-*O*-MeU, poly 2′-*O*-methyluridylic acid; poly 2′-*O*-MeA, poly 2′-*O*-methyladenylic acid; a double-stranded helical complex, such as that formed between poly rI and poly 2′-*O*-MeC is denoted by poly rI : 2′-*O*-MeC.

7.14×10^3 in 10^{-3} M-tris buffer, pH 8.0, and a sedimentation constant $S_{20,w}$ of 6.83 in 0.1 M-phosphate buffer, pH 7.8.

Poly rI, prepared from IDP (Sigma Chemical Co., St. Louis, Mo., U.S.A.) with *Escherichia coli* enzyme (Kimhi & Littauer, 1968), possessed an ε_{248} of 10.0×10^3 in 0.1 M-phosphate buffer, pH 7.8. Poly dI, obtained with the aid of terminal DNA nucleotidyl transferase (Bollum, 1966), was kindly supplied by Dr. F. J. Bollum; it had an ε^P_{260} of 5.35×10^3 in 10^{-3}M-tris buffer, pH 8.0, and an $S_{20,w}$ of 4.57 at pH 8.0.

UV absorption spectra, as well as melting profiles, were run on either a Unicam SP-500 or a Zeiss (Jena) VSU 2-P as elsewhere described (Żmudzka *et al.*, 1969a). Sedimentation constants were obtained with a Beckman Model E instrument fitted with ultraviolet optics. Measurements of pH made use of a Radiometer type 4d instrument with ordinary or semi-micro glass electrodes. Acetate buffers were used in the pH range 3.75 - 5.02, and phosphate buffers in the pH range 6.18 - 7.97, at a sodium ion concentration of 0.1 M. In what follows, the Na^+ concentration given includes the buffer cation concentration. Polynucleotide concentrations in various samples were maintained constant by weighing 1 g buffer to an accuracy of 10^{-4} g, followed by addition of the same volume of polymer solution under the same conditions.

RESULTS

Acid and neutral forms of poly 2′-O-MeC. It was previously shown (Żmudzka *et al.*, 1969c) that in acid medium poly 2′-*O*-MeC forms a structure similar to the partially protonated helical form of poly rC (Akinrimisi, Sander & Ts'o, 1963; Langridge & Rich, 1963); and preliminary findings demonstrated that this structure, like that of acid poly rC, differs in several respects from that for poly dC (Inman, 1964). We now present data which both confirm and extend these results.

Figure 1 presents the melting profile for poly 2′-*O*-MeC at pH values at which there are marked differences in properties between poly rC and poly dC. At about pH 4 both poly rC and poly dC are in the acid helical forms; but, whereas that for poly rC readily melts out by heating to 80°C, acid poly dC is unaffected even in the neighbourhood of 100°C. Heating of poly 2′-*O*-MeC leads to a melting out of the helical structure with a profile similar to that for poly rC. At a pH above 6 (right-hand side of Fig. 1) it is only poly dC which exhibits a cooperative melting profile typical of that for a helical structure; under these conditions poly rC and poly 2′-*O*-MeC show a small, approximately linear, increase in optical density on heating to 85°C, testifying to a non-cooperative "melting" of single-stranded polynucleotides.

A series of melting profiles was next run for poly 2′-*O*-MeC over the pH range 3.75 to 7.97. This demonstrated that, at room temperature in the presence of 0.1 M-Na^+, helical acid poly 2′-*O*-MeC begins to form at pH 4.9 (Fig. 2). Above pH 4.9 the melting of the polymer follows an approximately linear course (Fig. 3; see also right-hand side of Fig. 1), with an increase in optical density of about 15% at 270 nm, which is comparable to the 20% increase during melting of the single-stranded form of poly rC.

It is of some interest that, following completion of the cooperative melting of

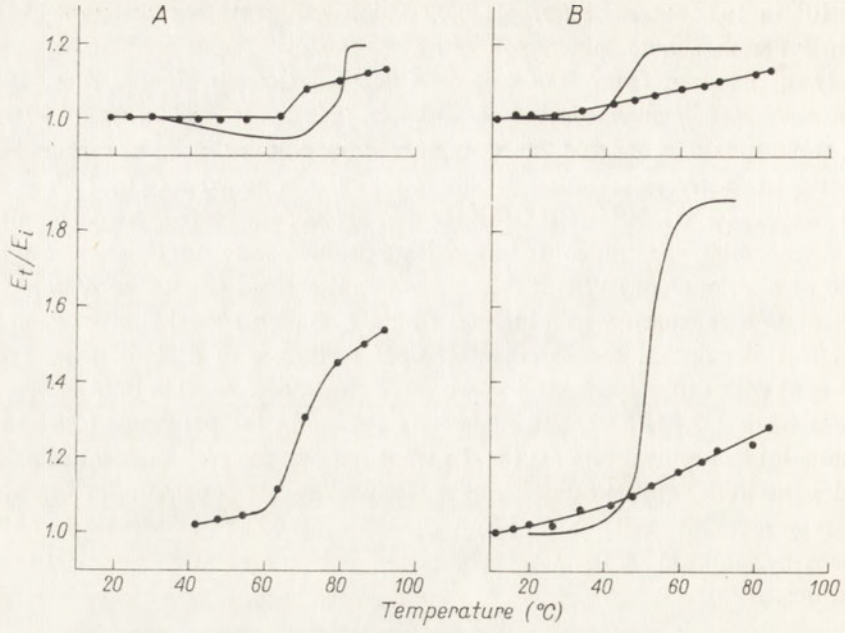

Fig. 1. Melting profiles (measured at wavelengths indicated) for poly 2′-*O*-MeC (-●-●-) as compared to *A*, poly rC and *B*, poly dC (——):

A, *upper frame*: poly rC, at 274 nm, 0.1 M-Na^+, pH 4.19 (taken from Szer & Shugar, 1966); poly 2′-*O*-MeC, at 272 nm, 0.1 M-Na^+, pH 4.20; *lower frame*: poly 2′-*O*-MeC, at 245 nm, 0.1 M-Na^+, pH 4.20 (there are no data in the literature for poly rC at 245 nm).

B, *upper frame*: poly dC, at 270 nm, 0.41 M-Na^+, pH 6.90 (taken from Inman, 1964); poly 2′-*O*-MeC, at 268 nm, 0.1 M-Na^+, pH 6.18; *lower frame*: poly dC, at 240 nm, 0.42 M-Na^+, pH 6.57 (taken from Żmudzka *et al.*, 1969a); poly 2′-*O*-MeC, at 250 nm, 0.1 M-Na^+, pH 6.18.

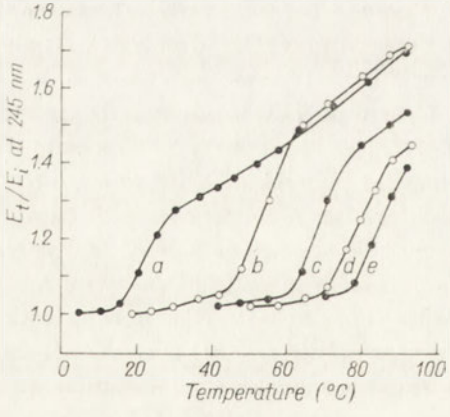

Fig. 2

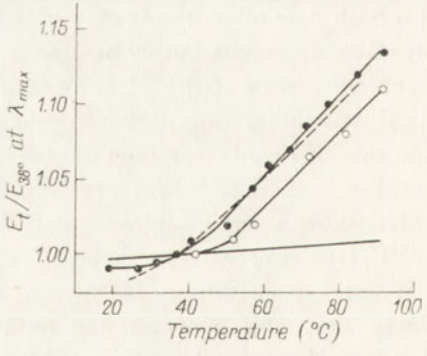

Fig. 3

Fig. 2. Melting profiles for poly 2′-*O*-MeC in 0.1 M-Na^+ at *a*, pH 4.90; *b*, pH 4.60; *c*, pH 4.20; *d*, pH 3.90; *e*, pH 3.75.

Fig. 3. Melting profiles of non-cooperative structural forms, measured at λ_{max}, for poly 2′-*O*-MeC in 0.1 M-Na^+, pH 7.97 (-●-●-); and pH 4.90 (-○-○-); for poly rC in 0.1 M-Na^+, pH 7.8, taken from Szer & Shugar (1966) (— — —); and for poly dC in 0.1 M-Na^+, pH 7.8 (——).

the helical form of poly 2′-*O*-MeC, there is a further increase in optical density with temperature which is virtually linear (Fig. 2). An analogous behaviour has been reported for acid helical poly rC (Brimacombe, 1967). The linear increase in optical density above 38°C at pH 4.90 (curve *a* of Fig. 2), expressed as the relative increase in optical density with respect to that at 38°C (end-point of the cooperative melting profile) was found to approximately parallel the non-cooperative increase in optical density accompanying the "melting" of the single-stranded form of poly 2′-*O*-MeC at about neutral pH at λ_{max}, 270 nm (Fig. 3) or at 245 nm (not shown in figure). This appears at first sight to indicate that, following melting out of the helical structure of acid helical poly 2′-*O*-MeC at pH 4.9 and 38°C, the subsequent non-cooperative process, measured by the increase in absorption at 245 nm, is that for "melting" of the neutral form of single-stranded poly 2′-*O*-MeC. However, it should not be overlooked that, at acid pH, deprotonation of cytosine residues is accompanied by spectral changes. The spectral modifications accompanying thermally induced deprotonation of monomers of cytidine and CMP have been recorded by Wróbel, Rabczenko & Shugar (1970), and are hyperchromic at about 245 nm. The linear portions of the profiles in Fig. 2 should consequently be somewhat steeper than those for the neutral single-stranded form of poly 2′-*O*-MeC in Fig. 3. Close examination shows that this is, in fact, the case, but the difference is somewhat less than that anticipated.

Evidence for the existence of the acid helical form of poly 2′-*O*-MeC at pH 4.2, and the single-stranded neutral form at pH 6.18, is further provided by the UV absorption spectra at both pH values at room temperature and following heating above 80°C (Figs. 4 and 5). At pH 4.2 (Fig. 4) the spectrum is fully analogous to that for acid poly rC, with the characteristic pronounced thermal hyperchromicity at 245 and 270 nm, and slight hypochromicity to the red of 295 nm. At pH 6.18 (Fig. 5) the spectrum resembles that of the neutral, single-stranded poly rC and, on heating, shows only hyperchromicity over the entire wavelength range.

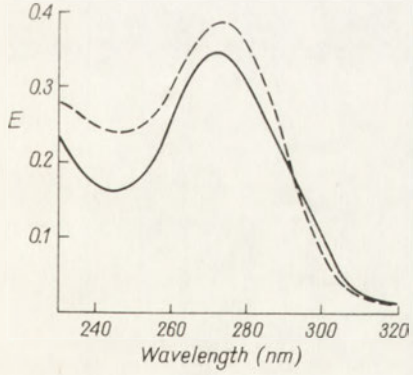

Fig. 4

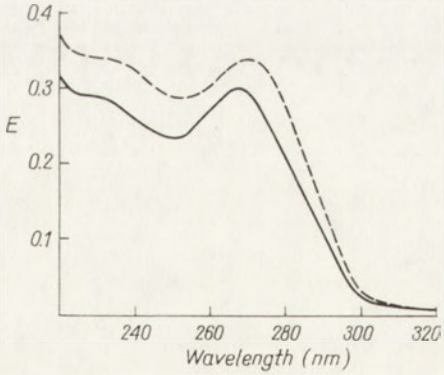

Fig. 5

Fig. 4. Absorption spectrum of poly 2′-*O*-MeC in 0.1 M-Na^+, pH 4.20 at 19°C (helical form, ——), and after melting out of co-operative structure, at 87°C (— — —).

Fig. 5. Absorption spectrum of poly 2′-*O*-MeC at pH 6.18 and 0.1 M-Na^+ at 11°C (——) and 80°C (— — —).

Figure 6 demonstrates the pH-dependence of the thermal stability of poly 2′-*O*-MeC along with the corresponding data for poly rC and poly dC. It will be noted that poly 2′-*O*-MeC, notwithstanding the fact that it does not possess free 2′-

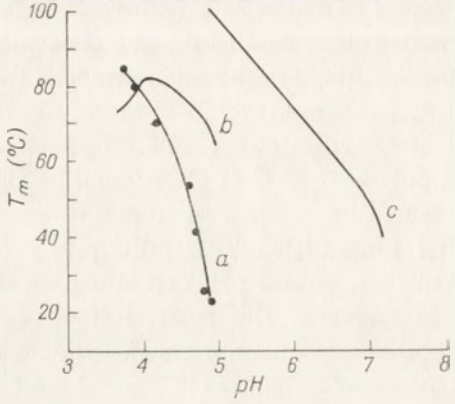

Fig. 6. pH-dependence, in the presence of 0.1 M-Na^+, of the T_m values of the acid forms of *a*, poly 2′-*O*-MeC; *b*, poly rC (from Akinrimisi *et al.*, 1963); *c*, poly dC (from Inman, 1964).

hydroxyls, is, like poly rC, in the acid form at pH values considerably different from those required for formation of acid poly dC, which makes its appearance at a pH value as elevated as 7.2.

Complexes with poly rI. The ability of poly 2′-*O*-MeC to complex with poly rI was examined by determining a series of melting profiles of 1:1 and 1:2 mixtures of these components at various Na^+ concentrations over the range 0.03 to 1.57 M (Fig. 7). In all instances the melting profiles were single-step in nature. The relatively high temperature hyperchromicity (58%) accompanying melting for the 1:1 mixture,

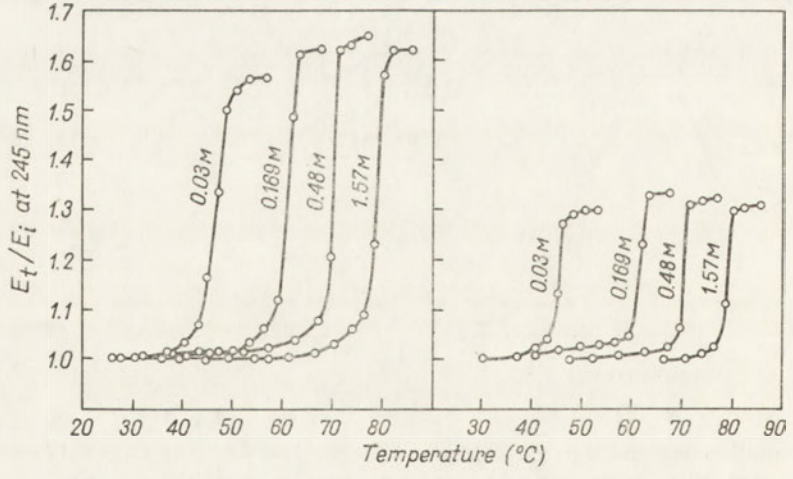

Fig. 7. Absorbance-temperature curves for mixtures of poly 2′-*O*-MeC and poly rI in molar ratios of 1 : 1 (left) and 1 : 2 (right) in 0.01 M-phosphate buffer, pH 7.8, and Na^+ concentration as indicated.

by comparison with the lower hyperchromicity (40%) observed for the 1:2 mixture of poly 2′-*O*-MeC with poly rI, pointed to the formation of only the double-stranded poly rI : 2′-*O*-MeC, irrespective of the ratio of the two components or of the ionic strength of the medium.

The absorption spectrum of this double-stranded helical structure is shown in Fig. 8, from which it will be noted that the hyperchromicity, relative to the sum of the individual components, is 58% at 245 nm and 7% at 265 nm. The absorption spectrum is similar to that for poly rI : rC; whereas the spectra of the complexes

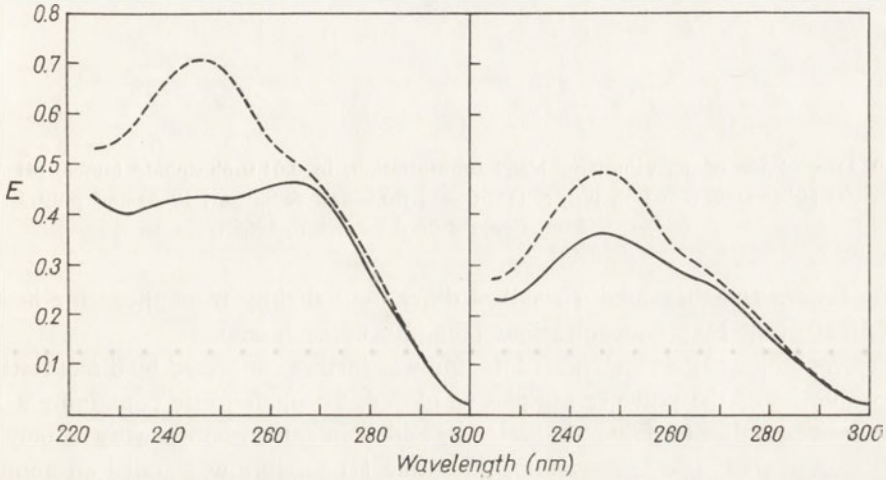

Fig. 8. Absorption spectra, in 0.01 M-phosphate buffer, pH 7.8, and 0.09 M-Na^+, of mixtures of poly rI and poly 2′-*O*-MeC at molar ratios of 1:1 (left) and 2:1 (right), at 24°C (helical forms, ——), and following melting to homopolymers at 65°C (— — —).

formed by poly dC, i.e. poly rI : dC and poly dI : dC, differ from the former (Chamberlin & Patterson, 1965) in that the hypochromicity at 245 nm is lower relative to that at 265 nm.

The dependence of the T_m values for poly rI : 2′-*O*-MeC on the Na^+ concentration is exhibited in the semi-logarithmic plot of Fig. 9. The melting temperatures are approximately 4°C lower, and 7°C higher, than the corresponding values for poly rI : rC and poly rI : dC respectively.

Complexes with poly dI. Attempts to obtain complexes of poly 2′-*O*-MeC with poly dI were carried out, as in the case of poly rI, by preparing 1:1 and 1:2 mixtures of the two. Rather surprisingly, mixing of the two components did not result in a decrease in optical density with respect to the arithmetic sum for the two when the Na^+ concentration was progressively increased from 0.02 to 0.23 M. A further increase in Na^+ concentration revealed a small drop in optical density at 0.48 M; but this did not attain the magnitude anticipated for complex formation even when the Na^+ concentration was raised to 0.94 M. The temperature profiles for these mixtures, presented in the upper portions of Fig. 10, demonstrate that the observed modifications in optical density are due, not to complex formation between poly 2′-*O*-MeC and poly dI, but to the formation and melting of the self-structure

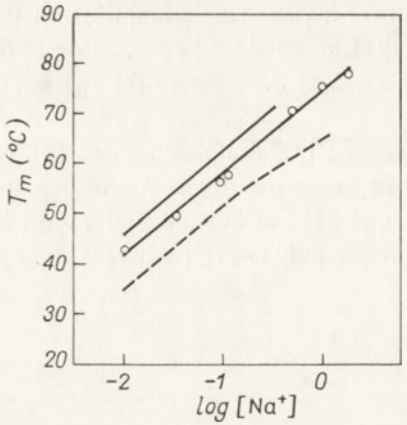

Fig. 9. Dependence of T_m values on NaCl concentration, in 0.01 M-phosphate buffer, pH 7.8, for poly rI : 2′-*O*-MeC (-○-○-), poly rI : rC (——, from Szer & Shugar, 1966) and poly rI : dC (— — —, from Chamberlin & Patterson, 1965).

of the latter. The measured T_m values differ only slightly from those for helical poly dI at given Na^+ concentrations (Fig. 10, upper insert).

The foregoing rather unexpected finding was further confirmed by direct tests of the properties of the polymer samples employed. To one cuvette containing a 1:1 mixture of poly dI and poly 2′-*O*-MeC was added an equimolar quantity of poly rI; while to a second cuvette containing the same 1:1 mixture was added an amount of poly dC equivalent to the amount of poly dI present (as indicated in Fig. 10). Each of the polymer solutions was then subjected to a heating cycle. The lower portions of Fig. 10 show in the first cuvette the typical melting profile for poly rI : 2′-*O*-MeC with a T_m of 76°C in agreement with the data from Fig. 9, as well as the melting profile for poly dI with a T_m of about 43°C, as observed in the same cuvette prior to addition of poly rI; and in the second cuvette a melting profile with a T_m of 55°C which is in agreement with literature data for the T_m of poly dI : dC at this salt concentration (insert to lower portion of Fig. 10).

DISCUSSION

As pointed out in the introduction, poly 2′-*O*-MeC is a particularly interesting model system for comparison with poly rC and poly dC. This is perhaps most strikingly illustrated by an examination of the so-called acid forms. In acid medium the cytosine residues of poly rC undergo protonation, leading to formation of a twin-stranded helix in which cytosine "base-pairs" are linked by two hydrogen bonds (involving one of the exocyclic amino hydrogens of each residue and the $C_{(2)}=O$ of the "complementary" residue) and an ionic bond between the ring $N_{(3)}$ nitrogens of both residues. The stability of this helix is maximal at a pH in the neighbourhood of the pK of the cytosine residues (p$K\sim4$) and rapidly decreases above pH 5 as a result of deprotonation. Poly dC likewise forms an acid twin-helical structure (Inman, 1964), but, for reasons hitherto not clear (see Żmudzka

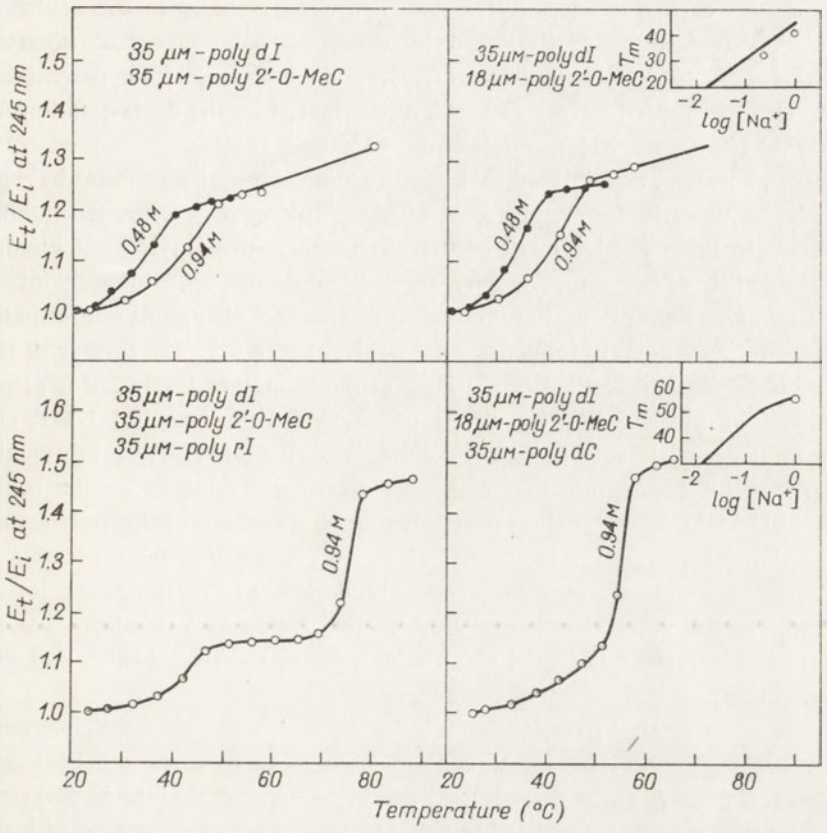

Fig. 10. Temperature-dependent modifications of optical densities of mixtures of poly dI : 2′-*O*-MeC, poly rI and poly dC at concentrations indicated in each figure, all in 0.1 M-phosphate buffer-pH 7.8, and Na^+ concentrations indicated beside each profile. *Inserts* show the dependence of T_m on Na^+ concentration for the self-structure of poly dI (upper insert) and the double-stranded poly dI : dC (lower insert); curves in both inserts are taken from Chamberlin & Patterson (1965), and the points are T_m values taken from the profiles in the figures in which each insert is located.

et al., 1969a, for discussion), this structure readily forms at slightly alkaline pH (~pH 7.2) and exhibits increasing stability with decrease in pH so that at pH 5 it will not melt out even at 100°C. This property, together with the relatively low thermal and enzymic hyperchromicities of the neutral single-stranded form of poly dC with respect to poly rC, was interpreted in terms of a supplementary hydrogen bond involving the 2′-hydroxyl of poly rC as the donor (Ts'o *et al.*, 1966), a suggestion subsequently adopted by numerous investigators, the hydrogen bond acceptor being variously ascribed to the $C_{(2)}=O$ of the aglycone in the case of cytosine polymers, the ring $N_{(3)}$ in the case of purines, or to an adjacent phosphate oxygen.

The fact that poly 2′-*O*-MeC, in which such hydrogen bond formation is excluded, forms a similar ordered structure at a pH even more acid than does poly rC (see Fig. 6), and with physico-chemical properties so different from poly dC, raises

serious doubts as to the necessity of such hydrogen bonding in the acid form of poly rC. These doubts are enhanced by the previously reported observations (Żmudzka *et al.*, 1969c) on the thermal (15%) and enzymic (28%) hyperchromicities for the neutral form of poly 2′-*O*-MeC, as compared to the corresponding values for poly rC (20% and 38%) and poly dC (2% and 18%).

It must, however, be admitted that the foregoing comparison cannot be regarded as conclusive in excluding involvement of the 2′-hydroxyl in hydrogen bonding in polyribonucleotides. Replacement of the 2′-OH by 2′-*O*-methyl, while eliminating any possible role of the 2′-OH as a hydrogen bond donor, unfortunately introduces at least two additional potential modifying factors: (a) change in conformation of the ribose residue, and (b) influence of the solvent medium. The former of these is susceptible to direct investigation, at least at the monomer level, and is at present the subject of investigation by means of circular dichroism and NMR studies.

The influence of the solvent itself is much more difficult to assess. It is, nonetheless, pertinent in this connection to recall some recent observations on the behaviour of poly 2′-*O*-MeU. The T_m values for the helix-coil transition of this polymer exhibit a linear dependence on the logarithm of monovalent cation concentration up to a value of the latter of 3 M. By contrast, the dependence of the T_m on log cation concentration for poly rU is linear only up to a value of 1 M; above 1 M, the T_m values decrease rapidly (Żmudzka & Shugar, 1971; Czuryło, Miller and Shugar, in preparation).

The observation that poly dU exhibits no thermal, and only negligible enzymic, hyperchromicity; and is unable under any conditions to form a helical complex (Żmudzka, Bollum & Shugar, 1969b), at first sight suggests that the helical structure formed by poly rU must necessarily be due to formation of some type of hydrogen bond involving the 2′-OH as donor. But poly 2′-*O*-MeU, in which such a hydrogen bond is not possible, forms a helical structure even more stable than poly rU (Żmudzka & Shugar, 1971).

It should be recalled that a pyrimidine 5-methyl substituent also enhances the stability of polynucleotides. For example, poly rT forms a helical structure with a considerably higher T_m than poly rU, and also forms complexes with poly A which are more stable than the corresponding complexes with poly rU (Szer, Świerkowski & Shugar, 1963; Barszcz & Shugar, 1968). A similar enhancement of stability is observed on replacement of the cytosine residues of poly rC by 5-methylcytosine (Szer & Shugar, 1966), or the cytosine residues of poly dC by 5-methylcytosine (Żmudzka *et al.*, 1969a). Even poly dT which, like poly dU, is incapable of forming a helical structure (Riley, Maling & Chamberlin, 1966), exhibits higher (albeit non-cooperative) thermal hyperchromicity than poly dU (Żmudzka *et al.*, 1969b). But this influence of a pyrimidine 5-methyl substituent is purely a quantitative effect (see Szer & Shugar, 1966 for full discussion), not comparable to that of a 2′-*O*-methyl.

The ability of poly 2′-*O*-MeC to form a double-stranded helix with poly rI might have been anticipated if it is recalled that both poly rC and poly dC likewise form such double-stranded helices with poly rI over a wide range of ionic strengths

and temperature. It is consequently all the more surprising that poly 2′-*O*-MeC is apparently unable to form such a helix with poly dI. It should, on the other hand, be noted that the double-stranded poly rI : 2′-*O*-MeC exhibits a T_m value lower than that of poly rI : rC, but higher than that of poly rI : dC (Fig. 9). It is conceivable that the decrease in T_m of the complex with poly rI accompanying introduction of the 2′-*O*-methyl in poly rC is the source of the inability to obtain an analogous complex with poly dI under our conditions. An examination of our own data, plus other culled from the literature, demonstrates the following, increasing order of thermal stability, in 0.1 M-Na^+, of various double-stranded complexes of I and C: dI+2′-*O*-MeC; dI : rC (35°C); dI : dC (46°C); rI : dC (52°C); rI : 2′-*O*-MeC (58°C); rI : rC (63°C). It will be seen that the lowest stabilities are exhibited by those helices which include poly dI, so that the introduction of the 2′-*O*-Me leads to a further reduction, with the result that no helix is formed.

In view of the rather close resemblance of poly 2′-*O*-MeC to poly rC, as compared to poly dC, based on physico-chemical considerations, it is of interest to inquire into the corresponding biological properties:

(a) *Interferon induction* ability of poly rI : 2′-*O*-MeC is practically nil when compared with poly rI : rC (De Clercq, Żmudzka and Shugar, in preparation). It has been suggested that this is due to the lower T_m of the foregoing, but this conclusion is questionable and is being further studied.

(b) *Messenger properties*: It has been demonstrated quite unequivocally that poly 2′-*O*-MeC is inactive as a messenger in an *in vitro* protein synthesis system, even in the presence of antibiotics (Price & Rottman, 1970; Dunlap, Friderici & Rottman, 1971).

(c) *Triphosphates as polymerase substrates*: No attempts have as yet been made to examine the properties of 2′-*O*-MeCTP as a substrate for RNA polymerases. However, Gerard, Rottman & Boezi (1971) have shown, with DNA-dependent RNA polymerase from *Pseudomonas putida*, that 2′-*O*-MeATP is bound by the polymerase and may undergo incorporation into the nascent RNA chain, although 90% of the 2′-*O*-MeA residues were located at the 3′-end of the chain. Furthermore, incorporation of a 2′-*O*-MeA residue into the nascent RNA chain limited subsequent chain growth. Although 2′-*O*-methyl residues are formed *in situ* by specific methylases in tRNA and rRNA, it would nonetheless be desirable to extend the above studies with the use of 2′-*O*-MeCTP as substrate, with a view to gaining further information as to the role of the 2′-hydroxyl in binding of substrates by polymerases. It undoubtedly would likewise be profitable to investigate the properties of 2′-*O*-methyl polynucleotides as potential templates.

We are very grateful to Mrs. E. Poddany for technical assistance. This investigation was supported by the Polish Academy of Sciences within the project 09.3.1 and also profited from the support of the World Health Organization, The Wellcome Trust and the Agricultural Research Service, U.S. Department of Agriculture.

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BADANIE STRUKTURY KWASU POLI 2′-*O*-METYLOCYTYDYLOWEGO I JEGO KOMPLEKSÓW Z KWASEM POLIINOZYNOWYM

Streszczenie

1. W środowisku obojętnym i alkalicznym kwas poli 2′-*O*-metylocytydylowy (poli 2′-*O*-MeC) występuje w formie pojedynczej nici, wykazującej podobieństwo do jednopasmowej formy poli rC, a nie poli dC.

2. W środowisku kwaśnym poli 2′-*O*-MeC tworzy dwupasmowy heliks o właściwościach podobnych do poli rC, a wyraźnie różnych od kwaśnej, heliksowej formy poli dC.

3. Poli 2′-*O*-MeC tworzy dwupasmowy heliks z poli rI. T_m dla przejścia heliks-kłębek tego kompleksu jest pośrednie między wartościami T_m dla kompleksów poli rI z poli rC lub poli dC.

4. Badano zależność stabilności kompleksu poli rI : 2′-*O*-MeC od siły jonowej.

5. Poli 2′-*O*-MeC w eksperymentalnie dostępnych warunkach temperatury i siły jonowej nie tworzy kompleksu z poli rI.

6. Przedyskutowano podobieństwo właściwości fizyko-chemicznych poli 2′-*O*-MeC i poli rC, częściowo w związku ze znanymi dużymi różnicami w ich aktywności biologicznej.

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RECENZJE KSIĄŻEK

W. B. Turner, FUNGAL METABOLITES. Academic Press, New York, London 1971; str. 446+IX, cena £ 9.—, $ 25.—

Fungal metabolites have been classified by W. B. Turner in a code-like way in a system based on biogenesis of secondary metabolites. This approach in contrast to classification based on chemical criteria reveals basic mechanisms of biosynthetic processes in secondary metabolism and explains interrelationship between metabolites of similar and divergent chemical structure. The main precursor of these metabolites is acetate, which is converted into: a) polyketides, b) secondary metabolites derived from fatty acids and c) terpenes and steroids. The secondary metabolites synthesized without intervention of acetate, Turner groups in classes of compounds derived from: 1) glucose or its catabolites, 2) shikimic acid, 3) amino acids, 4) intermediates of tricarboxylic acid cycle and 5) miscellaneous pathways.

The first two chapters of the book give general information on primary and secondary metabolites and short data on fungi and their cultivation. Although fungi are the main topic of the book, Turner largely quotes metabolites of plants and bacteria, mostly Streptomyces. A large list of references, and a supplement with the formula, organism, author, and subject indices make the book especially valuable as a reference offering a rapid, concise information. In addition the book serves another purpose — it gives an interesting, broad survey of biosynthetic pathways and mechanisms, including recent investigations, such as those on multienzyme systems. These two features of the book should make it popular among microbiologists, biochemists and pharmacists working on secondary metabolites.

Konstancja Raczyńska-Bojanowska

PROTIDES OF THE BIOLOGICAL FLUIDS. Proceedings of the Seventeenth Colloquium, Bruges, 1969 (H. Peeters, ed.). Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1970; str. 542, cena $ 18,75.

Omawiana książka stanowi zbiór materiałów 17-tej międzynarodowej konferencji poświęconej białkom płynów ustrojowych, która odbyła się w Bruges (Belgia) w 1969 r. Zawiera ona referat wprowadzający prof. J. F. Heremansa oraz 87 innych referatów i doniesień. W artykułach autorzy przedstawiają zarówno wyniki własnych badań, jak również dane z piśmiennictwa, zajmując w wielu wypadkach kontrowersyjne stanowisko.

W referacie pt. „Zmiany w cząsteczkach białkowych" Heremans wprowadza w zagadnienia, którym poświęcone jest całe sympozjum. Kolejno omówione są zmiany w cząsteczce białka, które autor dzieli na zależne od zmian w kodzie genetycznym (zmiany w sekwencji aminokwasów) i wtórne, zależne od warunków zewnętrznych powstałych i działających już po zakończeniu syntezy łańcucha peptydowego. Zmiany w strukturze białek zależeć więc mogą od zmian w konfiguracji cząsteczki, a także od sposobu połączenia poszczególnych łańcuchów; zmiany mogą powstać również przez modyfikację grupy prostetycznej, jak też na drodze interakcji białko-białko.

W sekcji A, najobszerniejszej (33 referaty), szeroko omówiono zagadnienia genetyki przeciwciał i ich ewolucji. Autorzy pierwszego w tej serii referatu wysuwają hipotezę, że u bezkręgowców mogą istnieć prekursory lekkich i ciężkich łańcuchów immunoglobin nie wykazujące typowych

funkcji, lub też białka, które nie są immunoglobinami, np. enzymy, obok swojej roli zasadniczej mogą spełniać również rolę przeciwciał. W następnych artykułach autorzy omawiają różne formy ludzkich immunoglobin, szukając w badaniach nad kodem genetycznym wytłumaczenia heterogenności tych białek. Ciekawą wydaje się propozycja, aby użyć heterogennych form immunoglobin jako modelu do badania procesów odczytywania kodu genetycznego (C. Milstein). L. Gyenes w artykule ,,Odwrócona teoria wahadła" w celu wyjaśnienia różnic w sekwencji aminokwasów w łańcuchach przeciwciał proponuje hipotezę udziału czwartego nukleotydu w kodzie mRNA, mianowicie inozyny. Stwarzałoby to możliwość rozpoznania takiego kodu przez 2 lub 3 różne tRNA, co w konsekwencji pozwoliłoby na wbudowanie 2 lub 3 alternatywnych aminokwasów, a w wypadku dwu inozyn 6 - 8 aminokwasów. Mimo teoretycznych możliwości i łatwego wytłumaczenia niektórych zjawisk, hipoteza ta nie wychodzi na razie poza spekulacje. W dalszych referatach autorzy próbują wyjaśnić genetyczne mechanizmy niedoboru immunoglobin oraz ustalić warunki w jakich dochodzi do hypo- i agammaglobulinemii. Dwa referaty poświęcone są roli grasicy w powstawaniu zespołów niedoboru przeciwciał. Szczególnie u człowieka zespół ten przybierać może różne formy. Mechanizmy patogenezy tego zespołu, a raczej zespołów, dalekie są od wyjaśnienia, wiele jednak wskazuje na to, że grasica odgrywa istotną rolę w tych procesach (H. W. Hess i H. Cottier).

Sekcja B poświęcona jest badaniom nad heterogennością poszczególnych białek. Autorzy przedstawiają dotychczasowe wyniki badań nad zmianami w strukturze hemoglobiny, fibrynogenu i innych aktywnych biologicznie białek ustrojowych. Obok zmian wynikających z błędów genetycznych, opisane są zmiany, które powstały w czasie rozwoju. Na przykładzie hemoglobiny stwierdzono, że spotyka się różne specyficzne formy tego białka w różnych okresach rozwoju zwierząt. Zmiany te zależą i powstają pod wpływem bardzo zróżnicowanych warunków fizjologicznych podczas rozwoju (E. R. Huehns). Szeroko omówione zostały zmiany spotykane w strukturze fibrynogenu. Nie wyjaśniona pozostaje mikroheterogeniczna struktura ludzkiego fibrynogenu. Brak również odpowiedzi, czy istnieją zależności między zmianami strukturalnymi fibrynogenu i zmianami w krzepliwości krwi. To ostatnie zagadnienie oprócz znaczenia czysto teoretycznego ma również kapitalne znaczenie kliniczne.

Trzecia część książki (Sekcje C i D) poświęcona jest nowym metodom i technikom stosowanym we współczesnych badaniach nad strukturą i jednorodnością poszczególnych białek. Oddzielną pozycję w badaniach homogenności i identyfikacji białek zajmuje metoda ,,isoelectric focusing" w gradiencie pH. Znalazło to odbicie w omawianej książce, gdzie szereg artykułów poświęconych jest wyłącznie tej metodzie (Sekcja C). Metoda ta oferuje wiele korzyści: prosta aparatura, możliwość przeprowadzenia szeregu równoległych, porównywalnych eksperymentów w jednym czasie, bardzo mała ilość materiału koniecznego do badania, możliwość zautomatyzowania całej metody. Omówione zostały zarówno teoretyczne podstawy całej metody, jak i różnorodne jej zastosowanie do charakteryzacji i rozdziału białek, również w połączeniu z innymi metodami (,,electrofocusing" na żelu i kolumnach na skalę preparatywną). W kilku ostatnich artykułach omówiono rozdział białek przez filtrację na żelu.

Z uwagi na dużą ilość wiadomości teoretycznych i metodycznych książka jest bardzo wartościową pozycją nie tylko dla pracowników bezpośrednio zainteresowanych biochemią zjawisk odpornościowych, ale także dla szerokiego ogółu biochemików. Wartość książki podnosi bogata bibliografia podana przy każdym artykule.

Witold Sendecki

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS, Vol. IX, (R. T. Holman, ed.). Part 4 and Part 5, *Polyunsaturated Acids*. Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1970. Part 4, str. 132, cena 6.75 $; Part 5, str. 95, cena 6.00$.

Kolejne dwa zeszyty tego wydawnictwa dotyczą zagadnień nienasyconych kwasów tłuszczowych.

W zeszycie 4 zamieszczono artykuły: ,,Biosynteza nienasyconych kwasów tłuszczowych" (E. M. Stearns, Jr), ,,Współzależności między wielonienasyconymi kwasami tłuszczowymi i przeciw-

utleniaczami *in vivo*" (L. A. Witting), oraz „Rola wielonienasyconych kwasów tłuszczowych w diecie i metabolizmie człowieka" (L. Soderhjelm, H. F. Wiese i R. T. Holman). W pierwszym z nich zwrócono szczególną uwagę na mechanizmy regulacji biosyntezy kwasów tłuszczowych, na zagadnienia kompartmentalizacji tego procesu oraz na wpływ etiolowania, temperatury i tlenu na przebieg procesu syntezy i rodzaj wytwarzanego produktu. W drugim artykule omówiono mechanizmy procesów peroksydacji i autooksydacji kwasów tłuszczowych oraz nowsze badania nad wpływem tokoferolu na metabolizm wielonienasyconych kwasów tłuszczowych. W artykule trzecim omówione są genetycznie uwarunkowane zaburzenia metabolizmu wielonienasyconych kwasów tłuszczowych oraz wyniki badań nad wpływem diety i niektórych witamin na metabolizm tych związków u ludzi.

W zeszycie 5 zamieszczono dwa artykuły: „Wielonienasycone kwasy tłuszczowe o nieparzystej liczbie atomów węgla" (H. Schlenk) oraz „Wielonienasycone kwasy tłuszczowe — aktywność biologiczna i zapotrzebowanie" (R. T. Holman). W pierwszym z nich omówiono głównie zagadnienia metodyczne — metody analizy tych kwasów, ich syntezy chemicznej oraz otrzymywania ze źródeł naturalnych. Poruszono również zagadnienie biologicznej aktywności tych kwasów w związku z możliwością powstawania z nich prostaglandyn. Ostatni artykuł stanowi bardzo obszerny przegląd zagadnień związanych z aktywnością biologiczną, budową i metabolizmem wielonienasyconych kwasów tłuszczowych; m.in. zwrócono uwagę na rolę tych związków w strukturach błonowych oraz na ich możliwe efekty toksyczne w organizmie.

Oba zeszyty stanowią wyczerpujące i wszechstronne omówienie zagadnienia wielonienasyconych kwasów tłuszczowych i będą wartościową pozycją w laboratoriach zajmujących się chemią i biochemią lipidów.

Tadeusz Chojnacki

ADIPOSE TISSUE, REGULATION AND METABOLIC FUNCTIONS (B. Jeanrenaud & D. Hepp, eds.). G. Thieme Verlag, Stuttgart; Academic Press, New York, London 1970; str. 212; cena DM 42,-

Książka jest zbiorem 37 referatów z prac eksperymentalnych nad zagadnieniem regulacji metabolizmu komórki tłuszczowej. Prace te dotyczą głównie hormonalnej regulacji metabolizmu. Jak wiadomo, biochemiczne badania eksperymentalne nad izolowanymi komórkami tłuszczowymi pozwoliły wykazać, że komórki te są wrażliwe na działanie rozmaitych hormonów, jak hormon wzrostowy, hormon tarczycy, insulina, prostaglandyny i inne. Duże ożywienie w endokrynologii wywołane odkryciem roli cyklicznego AMP jest wyraźnie odnotowane w materiałach referatowych omawianej książki. Rolę cyklicznego AMP jako kluczowego związku w mechanizmie działania szeregu hormonów oraz wpływ tego nukleotydu na przemianę kwasów nukleinowych w komórkach tłuszczowych omawia duża część referatów recenzowanego zbioru. W artykułach dotyczących mechanizmu działania hormonów *via* cykliczny AMP przytacza się dowody na istnienie specyficznych dla danych hormonów receptorów w błonach plazmatycznych, które są gatunkowo specyficzne. Wyniki przedstawianych w referatach badań zwracają w związku z tym uwagę na istnienie genetycznie uwarunkowanych różnic w budowie błon plazmatycznych. Treścią pozostałych artykułów są zagadnienia dotyczące przemiany lipidów w tkance tłuszczowej oraz hormonalna regulacja tej przemiany. Omówione są również współzależności metaboliczne między tkanką tłuszczową a tkankami innych narządów. Zagadnienie metabolizmu tłuszczy w stanie głodzenia i w cukrzycy jest tematem przewijającym się w wielu referatach. Każdy z artykułów zaopatrzony jest w bogaty materiał dokumentacyjny oraz w spis cytowanej literatury.

Książka stanowić będzie cenną pozycję nie tylko dla biochemików-endokrynologów, lecz również dla wszystkich zajmujących się biochemią cyklicznego AMP oraz biochemią procesów różnicowania komórek.

Tadeusz Chojnacki

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS. Vol. XI, part 2 (R. T. Holman, ed.). P. G. Fast: *Insect Lipids*. A. M. Parsons: *Pesticide Residues in Fats and other Lipids*. Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1970; str. 114, cena 48/-, $ 6.50.

Omawiany kolejny XI tom wydawnictwa zawiera dwie monografie. Pierwsza z nich w zwięzły i przejrzysty sposób przedstawia obecny stan wiedzy o lipidach występujących w świecie owadów. W kolejnych rozdziałach omówione są wszystkie składniki lipidów. Autor daje obszerny przegląd rzadziej spotykanych nienasyconych kwasów tłuszczowych (14:1, 16:1, 18:1, 18:2, 18:3) występujących w znacznych ilościach obok kwasów nasyconych u ponad 200 gatunków owadów reprezentujących 11 rzędów, oraz przedstawia charakterystykę glicerydów, steroli i fosfolipidów. Spośród grupy fosfolipidów wiele uwagi poświęcono fosfatydylocholinie i fosfatydyloetanoloaminie oraz wzajemnym ich stosunkom w obrębie jednego gatunku, jak też dużym różnicom występującym między różnymi gatunkami owadów, co według autora można wiązać z ich strukturą chemiczną, zagadnieniem błon komórkowych i kinetyką enzymatyczną. Omówiono również występowanie nienasyconych kwasów tłuszczowych w tych związkach. W osobnym rozdziale zajęto się biosyntezą lipidów, a szczególnie biosyntezą kwasów tłuszczowych i fosfolipidów. Ze względu na występowanie w kutikuli wolnych kwasów tłuszczowych nasyconych i nienasyconych oraz ich estrów, autor omówił jej skład i budowę, na przykładzie kilku wybranych gatunków, w oparciu o hipotezę o ciekłych kryształach. Przedstawiono także wpływ temperatury na powstawanie obu grup kwasów tłuszczowych w warunkach zaadaptowania hodowli w wyższej lub niższej temperaturze otoczenia. W ostatnim rozdziale zreferowano występowanie różnych lipidów w przebiegu całego cyklu rozwojowego owadów. W zakończeniu autor zwraca uwagę czytelnika na sterującą rolę hormonu juwenilnego w syntezie i utlenianiu lipidów ciała tłuszczowego. Szczegółowe dane poparte licznymi tabelami i wykresami, zwięzła dyskusja przedstawionych wyników oraz 281 pozycji piśmiennictwa stawiają tę monografię w rzędzie podstawowych prac dla wszystkich pragnących pogłębić swą wiedzę w zakresie biochemii owadów.

Druga monografia dotyczy badań nad pestycydami, głównie z grupy węglowodorów chlorowanych. Autor wiele uwagi poświęca preparatom DDT, Lindanu, Heptachloru, Aldriny, Dieldriny, Endriny i in., podając ich szczegółową charakterystykę oraz metody wykrywania tych związków w różnych tkankach przy pomocy oznaczeń kolorymetrycznych i chromatograficznych techniką bibułową, cienkowarstwową, kolumnową i w fazie gaz-ciecz. Wszystkie wymienione preparaty są w różnym stopniu toksyczne, niezwykle trwałe i bardzo wolno ulegające rozkładowi, a ich produkty metabolizmu, nagromadzając się w różnych tkankach, stanowią poważne źródło zagrożenia dla organizmów żywych zarówno w środowisku naturalnym, jak i dla zwierząt hodowlanych, a przede wszystkim dla człowieka. Na podstawie danych z wielu krajów różnych kontynentów autor przedstawia wielkość skażenia, jakiemu w ostatnim 10-leciu ulegał człowiek (mg/kg wagi, mleko i tłuszcz), zwierzęta dzikie w morzach i na lądzie (tłuszcz i jaja) i zwierzęta hodowlane (mleko i masło) oraz oleje roślinne. Ponadto podane są dawki śmiertelne (LD_{50}) dla szczurów, oraz dopuszczalne skażenie produktów żywnościowych ustalone przez FAO/WHO. Liczny materiał dowodowy oparty na 387 pozycjach piśmiennictwa w przekonywający sposób przedstawia zagrożenie ze strony pestycydów.

Maria J. Piechowska

ADVANCES IN PARENTERAL NUTRITION (G. Berg, ed.) G. Thieme Verlag, Stuttgart 1970; str. 243, cena DM 39.-

Książka jest zbiorem 33 referatów (15 w języku angielskim, 18 w niemieckim) wygłoszonych na sympozjum International Society of Parenteral Nutrition w Pradze, we wrześniu 1969 r.

Referaty podzielone są na trzy grupy tematyczne. Grupa pierwsza obejmuje 12 prac poświęconych kompletnemu odżywianiu pozajelitowemu. Druga grupa, złożona z 10 prac, omawia stosowanie pozajelitowe aminokwasów u pacjentów z różnymi schorzeniami. Grupa trzecia, obejmująca 11 prac, poświęcona jest efektom fizjologicznym i klinicznym odżywiania pozajelitowego. Wszystkie referaty

wskazują, że na tym polu dokonał się olbrzymi postęp. Przedstawione prace powinny zainteresować przede wszystkim lekarzy oddziałów chirurgicznych i stacji intensywnej opieki, bowiem prace te zawierają wiele cennych wskazówek praktycznych. Dla polskich lekarzy niewątpliwie najciekawsze są prace omawiające różne efekty odżywiania pozajelitowego emulsjami tłuszczowymi, podawanymi dożylnie. Uzyskane wyniki są bardzo zachęcające i warte popularyzacji.

Omówiona książka jest cenną pozycją dla lekarzy praktyków i biochemików klinicznych i powinna znaleźć się w ich bibliotekach podręcznych.

Juliusz Popowicz

THIRD INTERNATIONAL MEETING OF THE INTERNATIONAL SOCIETY FOR NEUROCHEMISTRY. Budapest 5 - 9 July, 1971, Abstracts (J. Domonkos, A. Fonyo, I. Huszak & J. Szentagothai, eds). Akadémiai Kiadó, Budapest 1971.

Książka zawiera streszczenia około 300 doniesień i 39 referatów sympozjalnych (str. 104 - 142) z III Międzynarodowego Zjazdu Towarzystwa Neurochemicznego, który odbył się w dniach od 5 do 9 lipca 1971 roku w Budapeszcie.

Streszczenia poszczególnych prac nie przekraczają 50 wierszy druku i przeważnie zawierają piśmiennictwo. Pod każdym streszczeniem Redakcja umieszcza główne hasła tekstu (key words) w celu ułatwienia czytelnikowi klasyfikacji merytorycznej zebranych prac. Na końcu książki podany jest alfabetyczny spis autorów. Niestety, są to jedyne udogodnienia w korzystaniu z obszernego zbioru streszczeń. Przy bogatej i różnorodnej tematyce dotkliwie odczuwa się brak indeksu rzeczowego.

Zwraca uwagę duża ilość doniesień z prawie wszystkich krajów Europy, ze Stanów Zjednoczonych, Kanady, Australii, Argentyny oraz z Japonii. Z licznych poruszanych problemów wymienić mogę w tym miejscu tylko niektóre.

Wiele miejsca zajmują doniesienia związane z badaniami nad metabolizmem glukozy w mózgu (str. 9 - 15, 111, 139, 255).

Na stronach 32 - 40 oraz 197, 199 znajdzie czytelnik prace poświęcone metabolizmowi kwasów nukleinowych, biosyntezie RNA, preparowaniu aktywnych podjednostek rybosomalnych.

Szereg doniesień dotyczy biosyntezy kwasów tłuszczowych (str. 250), transportu choliny i jej włączania do fosfolipidów w zakończeniach nerwowych w mózgu (str. 247), wpływu acetylocholiny na metabolizm fosfoinozytolu w strukturach synaptycznych (str. 246) analizy tłuszczów występujących w błonach synaptycznych (str. 245), rozmieszczenia fosfolipazy we frakcjach podkomórkowych mózgu (str. 249).

Katecholaminom i enzymom związanym z ich metabolizmem poświęcone są prace na stronach 166, 172 oraz na stronach 320, 324, 330 - 335, 350, 440.

Tematem bardzo wielu streszczeń są badania nad enzymami w centralnym i obwodowym układzie nerwowym, jak również badania nad wpływem leków na metabolizm tkanki nerwowej.

Wydany zbiór doniesień nadesłanych na III Międzynarodowy Zjazd Towarzystwa Neurochemicznego, ze względu na ich dużą ilość, jak również na szeroki zakres tematów, pozwala zorientować się w aktualnej problematyce badawczej w dziedzinie neurochemii różnych ośrodków naukowych na świecie. Książkę można zatem polecić biochemikom, fizjologom i farmakologom, zajmującym się badaniami tkanki nerwowej, oraz neurologom i psychiatrom pracującym w klinikach.

Ina Gąsiorowska

HYPERLIPIDÄMIEN. KLASSIFIZIERUNG, UNTERSUCHUNGSMETHODIK, THERAPIE. Symposium in Erlangen am 20 und 21 März 1970 (G. von Berg, ed.) G. Thieme Verlag, Stuttgart 1971; str. 118, cena DM 32.

Książka zawiera referaty wygłoszone na sympozjum, które odbyło się w Erlangen w marcu 1970 r. Wokół zagadnienia hiperlipemii koncentrują się obecnie zainteresowania wielu autorów między innymi dlatego, że stanowią one naturalne modele doświadczalne w badaniu mechanizmów powstawania miażdżycy tętnic.

W trzynastu artykułach omawianej książki zawarte są aktualne dane dotyczące stanów hiperlipemii. W pierwszym z nich D. S. Fredrickson przedstawia kryteria podziału hiperlipemii pierwotnych — wrodzonych, dziedzicznych zaburzeń metabolizmu lipidów — na 5 głównych typów. Podział Fredricksona przyjęty jest obecnie z niewielkimi modyfikacjami na całym świecie. W innym artykule opisano przypadki hiperlipemii typu I — najrzadszej postaci tej grupy schorzeń.

Dwie prace dotyczą hiperlipemii wtórnych: pierwsza z nich omawia własności patologicznych lipoproteidów pojawiających się w surowicy przy zastoju żółci, druga dotyczy zmian w zakresie lipidów surowicy w alkoholizmie.

W czterech artykułach przedstawiono metody pozwalające rozróżnić poszczególne typy hiperlipemii. We wszystkich tych pracach, jak również we wstępnym artykule Fredricksona, główny akcent położono na stosowanie metod prostych, w miarę możności z uniknięciem ultrawirowania, które może być wykonane tylko w laboratoriach wyspecjalizowanych. W dziedzinie rozpoznawania hiperlipemii znajdujemy się jeszcze na etapie poszukiwania prostych i pewnych metod diagnostycznych.

Tylko jeden artykuł dotyczy zmian aktywności enzymów tkanki tłuszczowej jako ewentualnego podłoża hiperlipemii. Autorzy stwierdzili pewne zmiany w aktywności enzymów glikolizy i cyklu oksydatywnego, niestety nie zajmowali się zupełnie enzymami lipolitycznymi tkanki tłuszczowej.

W dalszych artykułach przedstawione są możliwości terapeutyczne: dietetyczne oraz farmakologiczne, dotyczące leczenia hiperlipemii samoistnych.

Omawianą publikację zaliczyć można do dziedziny biochemii klinicznej. Zajmuje się ona przede wszystkim praktyczną stroną zagadnienia hiperlipemii, mniej akcentując jego aspekty teoretyczne, które są zresztą w chwili obecnej mało poznane. Książka stanowi wraz z zawartym w niej piśmiennictwem wyczerpujące źródło informacji z dziedziny hiperlipemii samoistnych.

Hanna Wehr

PROTIDES OF THE BIOLOGICAL FLUIDS. Proceedings of the Eighteenth Colloquium, Bruges, 1970 (H. Peeters, ed.). Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1971; str. 575; cena £ 9.50, $ 24.50.

Kolejny 18 tom serii *Protides of the Biological Fluids*, zawierający materiały z międzynarodowego sympozjum w Bruges (Belgia), poświęcony jest kilku tematom wybranym z rozległej dziedziny chemii białek, oraz najnowszym metodom służącym badaniu białek.

W części pierwszej można wyróżnić następujące grupy zagadnień: różnorodność struktur białkowych; białka płodowe, białka występujące we krwi pępowinowej; immunoglobuliny występujące u noworodków; współzależność między dojrzewaniem centralnego układu nerwowego a jego białkami.

W części drugiej znajdujemy artykuły poświęcone występowaniu w surowicy, w późniejszych okresach rozwoju ontogenetycznego, białek charakterystycznych dla okresu płodowego — co jest związane z powstawaniem pewnych chorób nowotworowych. Zjawisko to ma kolosalne znaczenie praktyczne, ponieważ przy zastosowaniu odpowiednich technik immunobiologicznych można wykryć wczesne stadia chorób nowotworowych. Tematycznie część druga jest podzielona na trzy grupy referatów: występowanie białka płodowego u osobników z rakiem wątroby; powstawanie specyficznych immunoglobulin w przebiegu chorób nowotworowych przewodu pokarmowego; zmiany występujące w białkach surowicy w przebiegu nowotworów układu krwiotwórczego.

W części trzeciej znajdują się prace zajmujące się wzajemnym oddziaływaniem cząsteczek białkowych w procesie agregacji i asocjacji miozyny, fibryny i kazeiny. Autorzy kilku prac poświęcili szczególną uwagę haptoglobulinom, ich właściwościom fizyko-chemicznym, metodom otrzymywania i ilościowego oznaczania.

W części poświęconej metodom omówione są najnowsze osiągnięcia w dziedzinie otrzymywania i oczyszczania białek, głównie enzymatycznych, które wskutek pewnych specyficznych reakcji

stały się nierozpuszczalne. Omówione są również nowe metody otrzymywania i oczyszczania przeciwciał.

Całość poprzedzona jest wykładem H. Peetersa na temat wzajemnej zależności i funkcji części białkowej i tłuszczowej lipoproteidów.

Książka wydana jest bardzo starannie. Prace, ilustrowane bardzo licznymi tabelami, wykresami i zdjęciami, uwzględniają najnowszą literaturę. Książkę zamyka spis autorów i indeks rzeczowy. Pozycja ta może być bardzo użyteczna dla wszystkich zajmujących się białkami płynów ustrojowych.

Marek Ombach

HAZARDS IN THE CHEMICAL LABORATORY (redaktor G. D. Muir). Royal Institute of Chemistry, London 1971; str. 266, cena £ 2.00.

Książka, której tytuł w polskim tłumaczeniu brzmi: *Niebezpieczeństwa w pracowniach chemicznych*, została wydana przez Royal Institute of Chemistry pod redakcją dr G. D. Muira, który równocześnie jest jej współautorem. Jest to nowe, rozszerzone opracowanie „*Laboratory Handbook of Toxic Agents*", wydanego w r. 1960. W recenzowanej książce oprócz czynników toksycznych zostały omówione również niebezpieczeństwa wynikające z użycia związków palnych, zakażenia w szpitalnych laboratoriach biochemicznych oraz zagrożenia promieniowaniem.

Rozdział 1 - *Wprowadzenie i ogólne zasady* (S. G. Luxon). Autor we wstępie bardzo słusznie zwraca uwagę, że ludzie — nie wyłączając chemików — nawet zdając sobie dobrze sprawę z możliwości nieszczęśliwych wypadków, uważają na ogół, że im osobiście nie może się nic przydarzyć, ponieważ są zbyt mądrzy na to, aby zrobić takie „głupie" pomyłki, jakie zrobili inni. Prowadzi to często do lekceważenia podstawowych zasad ostrożności, a w konsekwencji do chronicznych zatruć lub wypadków.

Następnie autor krótko omawia różne rodzaje niebezpieczeństw grożących w laboratoriach chemicznych i biochemicznych.

Rozdział 2 - *Planowanie bezpiecznych warunków pracy* (J. S. Beard i E. N. Dodd). Mowa tu o użyciu odpowiedniego materiału do wyposażenia laboratorium i o organizacji stanowiska pracy; podano też ogólne wytyczne i specjalne zasady ostrożności. Specjalną uwagę zwrócono na rękawiczki ochronne wykonane z różnych materiałów, odpornych na rozmaite substancje żrące.

Rozdział 3 - *Ochrona przed pożarem* (G. C. Acroyd, H. D. Taylor i M. Sheldon). Autorzy omawiają różne zagrożenia pożarowe, podają sposoby zapobiegania pożarom oraz metody gaszenia ognia.

Rozdział 4 - *Pierwsza pomoc* (G. D. Muir). Rozdział ten podaje wytyczne postępowania przy oparzeniach i zatruciach. Sztuczne oddychanie metodą tradycyjną i metodą usta-usta ilustrowane jest rysunkami.

Rozdział 5 - *Niebezpieczne gazy, odczynniki i rozpuszczalniki* (G. D. Muir). Rozdział ten jest najobszerniejszy (70% tekstu) i chyba najważniejszy. Autor podaje w kolejności alfabetycznej 430 związków lub grup związków szkodliwych dla człowieka a często używanych w przemyśle, pracowniach naukowych i studenckich. Po krótkiej charakterystyce każdego związku (wygląd, zapach, punkt wrzenia, rozpuszczalność) przedstawiono jego szkodliwe działanie i niebezpieczne własności. Następnie podano wskazówki pierwszej pomocy, ewentualnie sposób gaszenia pożaru. Na podkreślenie zasługuje forma i zwięzłość informacji, co umożliwia szybkie korzystanie z nich w razie wypadku. Zamieszczono również bardzo użyteczne dane, w jaki sposób należy usunąć i unieszkodliwić rozlaną lub rozsypaną substancję.

Cały ten rozdział wydrukowany jest na żółtym papierze, co ułatwia odróżnienie go od pozostałego tekstu.

Wydaje się, że brak jest wzorów chemicznych przy nazwach związków, co może sprawiać pewną trudność dla osób korzystających z książki, a nie mających w codziennym użytku nazw angielskich.

Rozdział 6 - *Bezpieczeństwo w szpitalnych pracowniach biochemicznych* (D. W. Neill i J. Russel Doggart). Wzięto tu pod uwagę pracownie analityczne i omówiono sposoby ochrony przed zakażeniem.

Rozdział 7 - *Ochrona przed promieniowaniem* (S. B. Osborn). Podano rodzaje promieniowania i środki bezpieczeństwa w pracy przy rentgenie i z radioizotopami.

Książka zasługuje na wysoką ocenę. O ile mi wiadomo, w polskiej literaturze nie mamy podobnej pozycji. Uważam, że należałoby jak najszybciej przetłumaczyć ten cenny podręcznik, który powinien znaleźć się w każdej pracowni chemicznej i biochemicznej, tak przemysłowej, jak i naukowej lub analitycznej.

Irena Mochnacka

WHO EXPERT COMMITTEE ON YELLOW FEVER: Third Report. World Health Organization Technical Report Series, 1971, no. 479; str. 56, cena 30 p, $ 1.00, Sw. fr. 3.-

Trzecie sprawozdanie Komitetu Ekspertów Światowej Organizacji Zdrowia dotyczące żółte febry przedstawia epidemiologię tej choroby, ekologię i charakterystykę wirusa, niebezpieczeństwo powstania epidemii oraz metody zapobiegawcze (szczepienia, zwalczanie organizmów będących nosicielami). Szczególną uwagę zwrócono na potrzebę wczesnego identyfikowania przypadków choroby w celu uniknięcia epidemii oraz omówiono trudności w postawieniu diagnozy w początkowym okresie choroby.

Publikacja powyższa została wydana również po francusku, a wydania rosyjskie i hiszpańskie są w przygotowaniu.

W Polsce wydawnictwa Światowej Organizacji Zdrowia można nabyć w Składnicy Księgarskiej (Warszawa, ul. Mazowiecka 9), a czasopisma za pośrednictwem Biura Kolportażu Wydawnictw Zagranicznych „Ruch" (Warszawa, Wronia 23).

Redakcja