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CONTENTS

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PURIFICATION AND PROPERTIES OF NUCLEASES FROM PIG PITUITARY GLAND

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1. From pig pituitary gland a partially purified preparation was obtained which contained both DNase and RNase activity. 2. This preparation on electrofocusing gave two fractions, which both contained the two activities. 3. The different effects of activators and temperature on the DNase and RNase activities, as well as the difference in the kinetics of DNA and RNA degradation, suggested that the two activities were associated with distinct proteins. 4. DNase was the more heat-labile enzyme. Its activity was somewhat enhanced by 1 mM-EDTA and Ca^{2+}, and was strongly inhibited by 1 mM-iodoacetate. The pH optimum was 5.5. Deoxynucleoside-3'-phosphates were found to be the only products of the partial and complete digestion of DNA. 5. RNase acted both in the presence or absence of divalent cations, and EDTA had no effect on its activity.

In the preceding paper, purification of RNase from pig pituitary by DEAE-Sephadex and CM-Sephadex column chromatography has been reported (Petryniak & Lisowski, 1968). Several active fractions were obtained, of which the 45-fold purified fraction *a''* contained 24% of the initial RNase activity and was inactive towards DNA.

As crude pig pituitary extracts were found to be active also towards DNA, an attempt was made to investigate the DNase activity.

MATERIALS AND METHODS

Chemicals. For the assay of DNase activity, highly polymerized calf thymus DNA (P.O.Ch., Gliwice, Poland) and sodium salt of calf thymus DNA, type V (Sigma Chem. Comp., St. Louis, Mo., U.S.A.) were used. To obtain denatured DNA, the aqueous solution of the DNA preparation containing 0.7 μmol of DNA phosphorus per 1 ml, was incubated at 100°C for 10 min, and then chilled rapidly.

Low-molecular yeast RNA was from B.D.H. (Poole, Dorset, England); to remove oligonucleotides, the aqueous solution of RNA was submitted to ultrafiltra-

tion in a dialysing tube (Visking, Serva, Heidelberg, G.F.R.). The solution was then dialysed against water and lyophilized.

Sodium salts of dAMP and dGMP, disodium salt of TMP, and trisodium salt of CDP were purchased from Sigma; dCMP was from Loba-Chemie (Wien-Fischamend, Austria). Deoxynucleoside-3'-phosphates were obtained by digestion of low-molecular fraction of DNA. To obtain this fraction salmon sperm DNA (B.D.H.) was dissolved in water, submitted to ultrafiltration, the filtrate lyophilized and digested with bovine spleen phosphodiesterase (Koch-Light Lab., Colnbrook, Bucks., England) as outlined by Hilmoe (1961). The reaction mixture (1.2 ml) containing 2.4 µmol of DNA-P, 100 µmol of Na-phosphate buffer, pH 7.0, and 200 µg of the enzyme, was incubated at 41°C for 90 min.

Bis(p-nitrophenyl)phosphate was a product of Calbiochem (Los Angeles, Calif., U.S.A.). CM-Sephadex C-50 was purchased from Pharmacia (Uppsala, Sweden). Carrier ampholyte, pH 6 - 8, was obtained from LKB (Stockholm-Bromma, Sweden).

Assay of DNase activity. The reaction mixture (2.5 ml) contained 0.7 µmol of native or denatured calf thymus DNA-P, 250 µmol of acetate buffer, pH 5.6, and the enzyme source corresponding to 0.6 - 1.0 unit of DNase activity. After incubation for 15 min at 41°C, the mixture was cooled in an ice bath for 2 min, then added with 0.5 ml of 1.28 M-perchloric acid, and after 10 min at 4°C the precipitate was removed by centrifugation. The extinction of the supernatant was read at 260 nm. An appropriate control without enzyme was run in parallel and its extinction was subtracted from that of the proper sample. One unit of DNase was defined as that amount of enzyme which under the above conditions and during 15 min gave an increase in extinction at 260 nm of 1.0.

Assay of RNase activity. The reaction mixture (0.5 ml) contained 4.0 µmol of RNA-P, 50 µmol of acetate buffer, pH 5.6, and the enzyme source corresponding to 1.5 - 3.0 RNase units. After incubation at 41°C for 15 min the reaction was stopped by addition of 0.5 ml of 0.48 M-trichloroacetic acid containing 0.19 M-uranyl acetate. After 10 min at 41°C, the precipitate was removed by centrifugation and 0.5 ml of the clear supernatant was usually diluted up to 2.5 ml with water and its extinction was read at 260 nm. An appropriate control without enzyme was run in parallel and its extinction was subtracted from that of the proper sample. One unit of RNase was defined as that amount of enzyme which gave an increase in extinction at 260 nm of 1.0. The reaction velocity was proportional to the amount of enzyme added up to 19% of the substrate utilized.

Assay of phosphodiesterase activity. The assay was carried out according to Koerner & Sinsheimer (1957) with the following modifications. The reaction mixture (3.0 ml) containing 3 µmol of bis(p-nitrophenyl)phosphate, 300 µmol of acetate buffer, pH 5.6, and 19 units of DNase activity was incubated for 15 min at 41°C, then the extinction at 440 nm was measured.

Electrofocusing was performed on the carrier ampholyte according to the LKB Instruction Manual. A column of 110 ml (LKB 8101) and a density gradient mixer (LKB 8121) were used.

Identification of DNA digestion products. The products were identified by paper chromatography and high-voltage electrophoresis using as standards deoxynucleoside-3'-phosphates and deoxynucleoside-5'-phosphates. Descending paper chromatography was carried out on Whatman no. 3 *MM* paper with isopropanol - water (70:30, v/v) and ammonia in vapour phase (Markham & Smith, 1952) for 54 h at room temperature. High-voltage electrophoresis was performed at room temperature on Whatman no. 3 paper in pyridine - acetic acid - water (1:10:89, by vol.), pH 3.5, at 44 V/cm for 100 min or in pyridine - acetic acid - water (6.2:5:88.8, by vol.), pH 5.0, at 16 V/cm for 2 h. For identification the spots were cut out from chromatogram or electrophoretogram and sewed together with a new sheet of paper, and another electrophoresis at appropriate pH was run. For quantitative analysis the spots were eluted with three 3-ml changes of 0.01 N-ammonia solution at 37°C for 14 h with gentle shaking. A control strip of paper was treated in the same way. The eluates were dried at 100°C and total phosphorus was estimated.

Total phosphorus was determined by the method of Bartlett (1959). The sample containing up to 0.3 μmol of phosphorus was dried before determination at 100°C. For colorimetric assay, disodium salt of EDTA was used.

Protein was determined by the procedure of Lowry *et al.* (1951) using ovoalbumin (Sigma) as standard.

<div align="center">RESULTS</div>

Purification of DNase and RNase. All operations were carried out at 4°C; buffers used for purification procedure contained 1 mM-EDTA-Na$_2$. Precipitates formed during dialysis of enzyme solutions were removed by centrifugation. The process of purification is summarized in Table 1.

Step 1. Fresh, frozen whole pig pituitary glands obtained from a slaughterhouse were ground in a tissue disintegrator and treated with 5 vol. of a cold solution of 0.1 M-ammonium sulphate containing 1 mM-EDTA-Na$_2$, and the mixture was adjusted to pH 7.3 with 2 M-NaOH. A few drops of toluene were added and the suspension was mechanically stirred overnight. The insoluble material was removed by centrifugation at 2100 g for 30 min.

Step 2. The supernatant was adjusted to pH 5.1 with 0.5 M-H$_2$SO$_4$, added with a few drops of toluene and incubated at 37°C for 24 h. A copious precipitate formed was removed by centrifugation.

Step 3. To the supernatant, EDTA-Na$_2$ was added to 1 mM concentration (37 mg/100 ml) and the solution was adjusted to pH 6.5 with 1 M-NaOH. Then powdered ammonium sulphate was added to 0.9 saturation (62.3 g/100 ml) with constant mechanical stirring within 30 min. The mixture was adjusted to pH 6.3 with 1 M-H$_2$SO$_4$. After 10 min the precipitate was collected by centrifugation, suspended in 1/6 volume of 0.1 M-acetate buffer, pH 5, and dialysed overnight against two changes of 10 vol. of the same buffer.

Step 4. The dialysed preparation was applied to the CM-Sephadex C-50 column equilibrated with 0.1 M-acetate buffer, pH 5 (Fig. 1). Three active fractions were obtained: *α* (tubes 70 - 120) with RNase activity, *β* (tubes 132 - 148) with RNase

Table 1

Purification of deoxyribonuclease and ribonuclease

Whole pig pituitary glands, 375 g, were used. The units of enzyme activity correspond to that amount of either enzyme which during 15 min under the applied conditions gave an increase of extinction at 260 nm of 1.0.

Purification steps	Volume (ml)	Total protein (mg)	DNase activity			RNase activity		
			total (units)	specific (units/mg of protein)	yield (%)	total (units)	specific (units/mg of protein)	yield (%)
1. Extract	1960	32 340	33 700	1	100	274 400	9	100
2. Autolysis at pH 5.1	1820	15 500	32 360	2	96	219 500	14	80
3. Ppt. at 0.9 ammonium sulphate sat.	444	6 300	23 950	4	71	193 200	31	70
4. Chromatography on CM-Sephadex C-50								
fraction α	663	1 400	0	0	0	22 200	16	8
fraction β	221	457	4 300	9	13	129 400	283	47
fraction γ	1196	780	9 580	12	28	0	0	0
5. Rechromatography of fraction β on CM-Sephadex C-50	119	200	4 300	21	13	116 500	583	42
6. Chromatography on CM-Sephadex C-50 (elution with pH gradient)	111	85	1 760	21	5	90 800	1 070	33

and DNase activities, and γ (tubes 149 - 240) with DNase activity only. For further purification only fraction β was used.

Step 5. Fraction β was concentrated to 1/5 vol. by ultrafiltration in Visking dialysis tubing under reduced pressure, and dialysed against two changes of 50 vol. of 0.1 M-acetate buffer, pH 5. After dialysis the preparation was rechromatographed on CM-Sephadex C-50 column equilibrated with 0.1 M-acetate buffer, pH 5 (Fig. 2). Active fractions (tubes 48 - 64) containing RNase and DNase activities, were pooled.

Step 6. Active fractions from the CM-Sephadex chromatography procedure were concentrated by ultrafiltration to 15 ml. The enzymic solution was dialysed against two changes of 40 vol. of tris - KH_2PO_4 - ammonium acetate buffer mixture (each reagent at final concn. of 0.02 M), pH 5.3. After dialysis the solution was
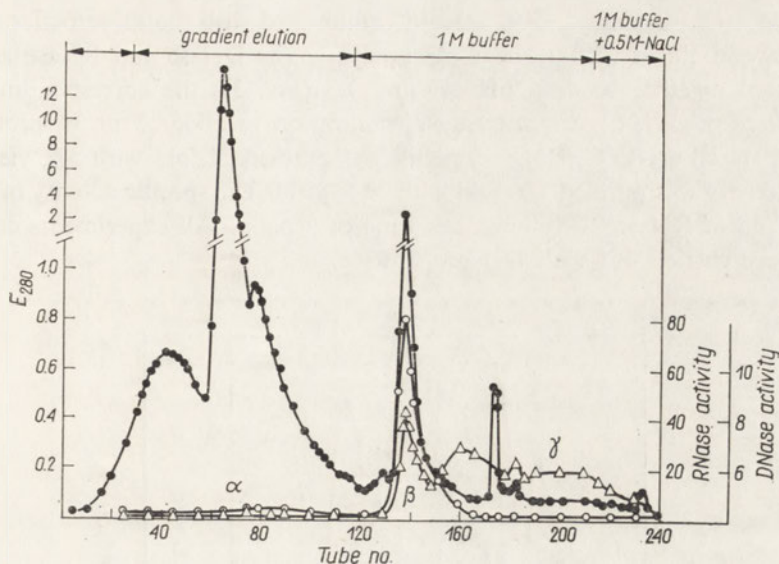
Fig. 1. Chromatography on CM-Sephadex C-50 of proteins precipitated with ammonium sulphate. The column (28.5×2.5 cm) was loaded with 2992 mg of protein (half of the amount obtained from step 3). A linear salt gradient was used for elution, with 250 ml of 0.1 M-acetate buffer, pH 5.0, in the mixing vessel and 250 ml of 1 M-acetate buffer, pH 5.0, in the reservoir. Then 600 ml of 1 M-acetate buffer, pH 5.0, followed by 150 ml of the same buffer containing 0.5 M-NaCl were passed through the column. Fractions of 6.5 ml were collected every 15 min. ●, Protein, E_{280}; △, DNase activity, units/ml; ○, RNase activity, units/ml.
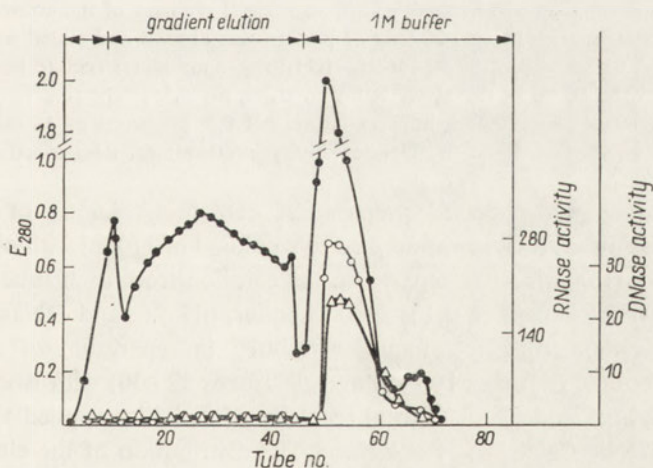
Fig. 2. Rechromatography of fraction β from Fig. 1 on CM-Sephadex C-50. To the column (19.0×1.6 cm) 228.5 mg of protein was applied. A linear salt gradient from 0.1 M to 1 M-acetate buffer, pH 5.0, was used for elution (total volume 140 ml). Then the column was washed with 90 ml of 1 M-acetate buffer, pH 5.0. Fractions of 3.5 ml were collected at 15 min intervals. ●, Protein, E_{280}; △, DNase activity, units/ml; ○, RNase activity, units/ml.

applied to a CM-Sephadex C-50 column equilibrated with the same buffer mixture as above, and eluted with a linear pH gradient. The DNase and RNase activities were eluted together between pH 5.7 and 7.3 (Fig. 3); the corresponding tubes 24 - 53 were pooled, concentrated by ultrafiltration to about 2 mg of protein per 1 ml and stored at -15°C. DNase activity was purified 21-fold with 5% yield, and RNase activity was purified 126-fold with 33% yield. The specific activity of DNase was 21, and of RNase 1070 units per 1 mg of protein. All experiments described below were carried out with this preparation.
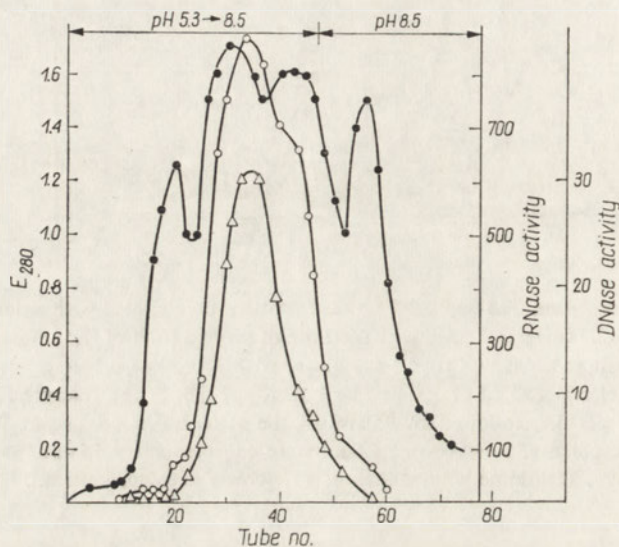
Fig. 3. Chromatography on CM-Sephadex C-50 with a pH gradient of the active fractions from step 5. To the column $(19 \times 1.6 \text{ cm})$ 200 mg of protein was applied and eluted with a linear pH gradient from 5.3 to 8.5 (80 ml of 0.24 M-tris - KH_2PO_4 - ammonium acetate buffer, pH 5.3, in the mixing vessel and 80 ml of the above buffer mixture, pH 8.5, in the reservoir). The gradient was followed by 80 ml of the 0.24 M buffer mixture, pH 8.5. Fractions of 3.7 ml were collected every 15 min. ●, Protein, E_{280}; △, DNase activity, units/ml; ○, RNase activity, units/ml.

Electrofocusing. The isolated preparation containing 8.6 mg of protein was dialysed against water, then it was applied to a column of 110 ml with carrier ampholyte of pH 6 - 8 (Fig. 4). After separation the solution from each tube was dialysed against two 3-litre changes of 0.1 M-acetate buffer, pH 5.6, and DNase and RNase activity were estimated. Electrofocusing resulted in separation of enzymes into two active fractions, β' (tubes 10 - 18) and β'' (tubes 22 - 30) with isoelectric points of, respectively, 6.5 and 5.8. The total recovery of activities applied to the column was 25% for DNase and 38% for RNase. The distribution of the eluted activities was as follows: DNase, 93% in fraction β' (sp. act. 17) and 7% in fraction β'' (sp. act. 0.8); RNase, 36% in fraction β' (sp. act. 517) and 64% in fraction β'' (sp. act. 580).

Properties of the isolated preparation. The obtained preparation was not active towards bis(*p*-nitrophenyl)phosphate.
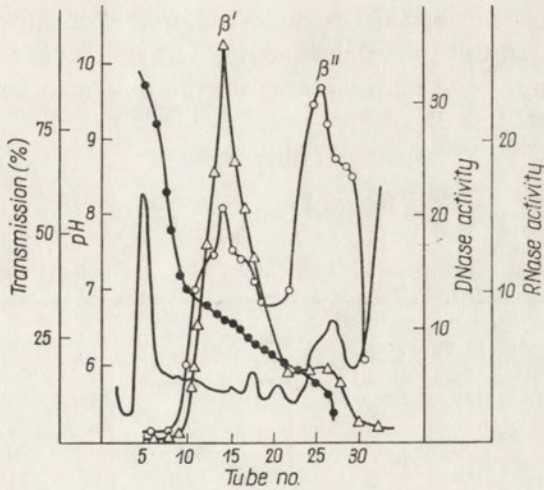
Fig. 4. Electrofocusing on the carrier ampholyte of pH 6-8 of the fraction from step 6. The column (110 ml) was loaded with 8.6 mg of protein. The running time was 44 h at 600 V; current decreased from 2.7 mA to 0.5 mA. Fractions of 3.6 ml were collected every 4 min, and pH of each fraction was measured. $\triangle$, DNase activity, units/fraction; $\bigcirc$, RNase activity, units/fraction; $\bullet$, pH gradient; ——, transmission at 254 nm.

The effect of some activators and inhibitors at 1 mM concentration on the RNase and DNase activity is presented in Table 2. EDTA, Mn^{2+} and Ca^{2+} did not affect RNase activity, whereas DNase activity was somewhat enhanced by EDTA and Ca^{2+}, and diminished by Mn^{2+}. Ag^{+} and iodoacetate inhibited both activities,

Table 2

The effect of activators and inhibitors on DNase and RNase activity

The assays were carried out as described in Methods. The reaction mixture contained 3.5 units of RNase (3 μg of protein) or 0.9 unit of DNase (43 μg of protein).

Addition (1 mM)	Substrate		
	RNA	Native DNA	Denatured DNA
	Activity (%)		
None	100	100	100
MgSO₄	90	96	90
EDTA (di-sodium salt)	100	127	114
MnSO₄	102	86	82
AgNO₃	60	0	0
CuSO₄	28	0	0
FeSO₄	95	90	76
CaCl₂	102	128	135
HgCl₂	0	0	0
NaN₃	105	107	103
CH₂ICOOH	84	20	20
NH₄F	91	94	86

however that of DNase to a greater extent. When, instead of native DNA, denatured DNA was used as substrate, the DNase activity was affected in a similar way.

Both RNase and DNase activities were thermolabile at pH 5.1, that of RNase being more stable (Table 3).

Table 3

Thermal stability of DNase and RNase

Solution of the preparation was dialysed against 0.1 M-acetate buffer, pH 5.1. After dialysis the samples containing 730 µg of protein in 100 µl were incubated for 5 min at the indicated temperature, then chilled to 4°C and adjusted to pH 5.6 by dilution with 0.1 M-acetate buffer, pH 5.6.

Incubation temperature (°C)	Activity (%)	
	DNase	RNase
20	100	100
40	84	100
60	37	83
80	0	11

DNA digestion. The optimum conditions for DNase activity were: a temperature range from 41° to 61°C (Fig. 5) and a pH value of 5.5 (Fig. 6).
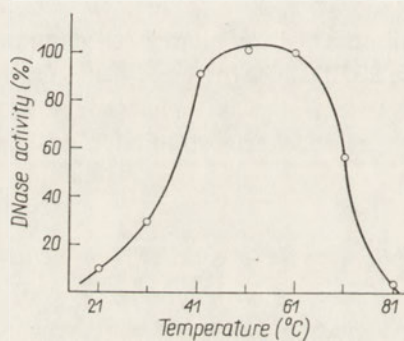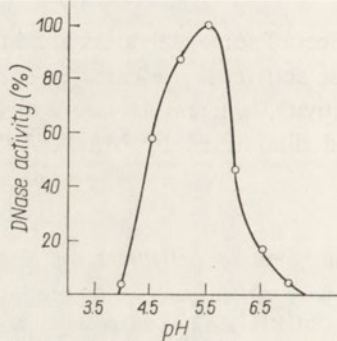
Fig. 5   Fig. 6

Fig. 5. Effect of temperature on DNase activity. Conditions as described in Methods.

Fig. 6. Effect of pH on DNase activity. The assays were performed in a buffer mixture composed of 0.1 M-tris - 0.1 M-KH$_2$PO$_4$ - 0.1 M-ammonium acetate, adjusted to appropriate pH with 0.3 M-acetic acid or 0.3 M-ammonia. The reaction mixture contained 0.5 ml of the buffer, 0.70 µmol of DNA-P, 0.9 DNase unit (43 µg of protein) and 2 ml of water. After incubation at 41°C for 15 min, 0.5 ml of 2.2 M-HClO$_4$ was added.

On incubation of 0.6 unit of DNase (29 µg of protein) with 0.7 µmol of DNA-P, after 27 min 88% of the substrate was hydrolysed to acid-soluble products (Fig. 7). The shape of the time-curve indicated substrate inhibition (see Laidler, 1958). This was confirmed by the effect of DNA concentration (Fig. 8). The reaction was linear up to 2.5 µmol DNA-P; at higher substrate concentrations, the reaction velocity decreased and eventually reached a plateau value corresponding to 1/3 of the maximum rate (not shown in the Figure).
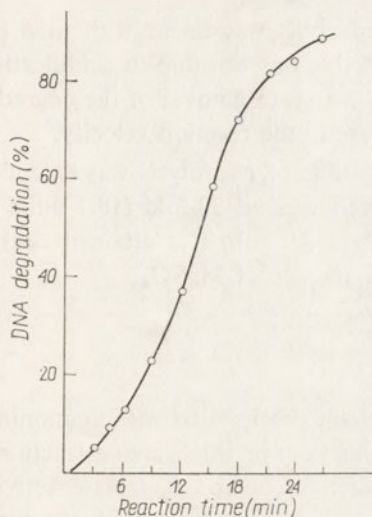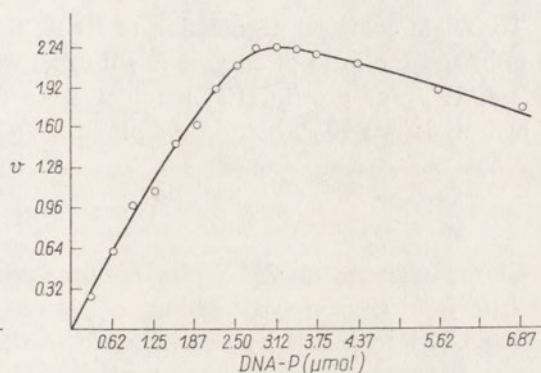
Fig. 7   Fig. 8

Fig. 7. Time-course of DNA hydrolysis. The reaction mixture contained 0.6 unit of DNase (29 μg of protein, 0.7 μmol of DNA-P). At the indicated times, samples were removed and total phosphorus was estimated in the acid-soluble products.

Fig. 8. Effect of DNA concentration on the reaction rate. The reaction mixture (4.5 ml) contained 6.0 units of DNase activity, 450 μmol of acetate buffer, pH 5.6, and the indicated amounts of DNA. The reaction was stopped by adding 0.5 ml of 2.2 M-HClO₄. v is expressed as μg of total acid-soluble phosphorus formed per 15 min.

For identification of the DNA digestion products, the incubation mixture (0.55 ml) contained 55 μmol of acetate buffer, pH 5.6, 1.9 μmol of DNA-P and 2.8 units of DNase. For partial digestion the incubation was carried out at room temperature for 20 min, and for complete digestion at 41°C for 45 min. After incubation, 300 μl of the incubation mixture was submitted to chromatography and electrophoresis at pH 3.5. In either sample only four spots were obtained during electrophoresis, with mobilities of standard deoxynucleoside-3′-phosphates. Quantitative analysis showed that in the first experiment 24% of DNA was hydrolysed, and in the second 100%.

RNA digestion. The time-course of decomposition of 2.7 μmol of RNA to acid-soluble products by 1.25 units of RNase (1.2 μg of protein), is presented in Fig. 9.

Fig. 9. Time-course of RNA digestion. The reaction mixture (1.5 ml) contained 150 μmol of acetate buffer, pH 5.6, 2.7 μmol of RNA-P, 15 μmol of MgSO₄ and 1.25 units of RNase. The samples were incubated at 41°C for the indicated time intervals. The reaction was stopped by adding 1.0 ml of 0.15 M-HClO₄ - 0.09 M-trichloroacetic acid - 0.036 M-uranyl acetate mixture. After 10 min at 41°C, the precipitate was centrifuged, dissolved in 4 ml of 0.01 M-NaOH, and the extinction at 260 nm was measured.
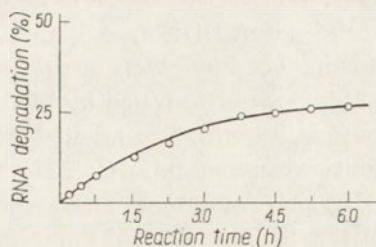
Within 6 h, 27% of the RNA was digested. Hydrolysis was linear with time for 45 min, then the rate of hydrolysis decreased but this was not due to acidification of the reaction mixture, as the pH value did not change. Removal of the degradation products by dialysis resulted in an 8% increase in the reaction velocity.

To obtain complete degradation of RNA, the time of hydrolysis was extended to 17 h, and the ratio of enzyme to substrate was increased 30-fold (18.7 units of RNase, 19 μg of protein, 1.35 μmol of RNA-P in 3.0 ml). The attained degree of hydrolysis was 99%, both in the presence and absence of $MgSO_4$.

DISCUSSION

Chromatography on CM-Sephadex of the proteins precipitated with ammonium sulphate from the autolysed extract of pig pituitary, gave three active fractions: a, β and γ. RNase activity was found in two fractions, a and β. DNase activity was present in fractions β and γ. Both these fractions were eluted with the same 1 M-acetate buffer, which may suggest that DNase activity in these fractions is related to two distinct enzymes.

An attempt was made to determine whether the RNase and DNase activities found in fraction β are associated with the same or distinct proteins. Although on further purification of fraction β the RNase and DNase activities were eluted together and their peaks were coincident, the ratio of the two activities changed. After chromatography on CM-Sephadex in the pH gradient (step 6) the ratio of DNase activity to RNase activity changed from 1:30 to 1:50. This observation suggested that RNase and DNase present in the preparation could be distinct proteins.

The electrofocusing technique failed to demonstrate directly the presence of two separate enzymes. The two active fractions obtained, β' and β'', still exhibited the dual mode of action, but the different elution profiles of the two activities indicated that both in fraction β' and β'' the RNase and DNase activities are associated with distinct proteins.

Differences in thermal stability and different effect of the inhibitors and activators on the activities of RNase and DNase in fraction β also confirmed this assumption.

Some properties of the investigated pituitary DNase preparation correspond to those of DNases of type II, which form 3'-monoesters (see reviews by Laskowski, 1961, 1967). The enzyme is similar to DNases II in that it hydrolyses native and heat-denatured DNA, and is active at acidic pH values. Like calf thymus DNase, it is inhibited by excess of substrate, and is activated by 1 mM-Ca^{2+}. On the other hand, it is not activated by Mg^{2+} and Mn^{2+} ion which activate both calf thymus and pig spleen DNases, and Mg^{2+} ion has even a slightly inhibitory effect on the activity of pituitary DNase. Since no attempt was made to remove Mg^{2+} from the DNA preparation used, it seems likely that the substrate could contain an amount of this ion which could diminish DNase activity. Stimulation of pituitary DNase

activity by EDTA supports this assumption. The pig pituitary DNase and pig spleen DNase differ in their isoelectric points, 6.5 and 10.2, respectively.

The investigated pituitary DNase differs strikingly from DNase II in its mode of action. Time-course of DNA digestion by spleen DNase exhibits two distinct phases: a rapid initial phase, when 10% of the total number of diester bonds are hydrolysed, and a very slow second phase, during which up to 25 - 30% of the diester bonds are broken. To increase the reaction velocity to the value observed during the first phase of the reaction, a 20-fold excess of enzyme is required. All DNase II-like enzymes are endonucleases and produce a mixture of mono-, di-, trinucleotides and higher oligonucleotides terminating in 3'-phosphate.

The pituitary DNase hydrolysed DNA to 3'-mononucleotides rapidly and completely. Among the products of partial and complete digestion we did not find fragments higher than mononucleotides. On the basis of these experiments it may be assumed that pituitary DNase is an exonuclease. However, the possibility of scission of the internal linkages in polydeoxyribonucleotide chain, with fragmentation into large oligonucleotides at the initial stages of DNA hydrolysis, cannot be excluded.

The mechanism of action of pituitary DNase, different from that of DNase II (*sensu stricto*), did not allow to classify it unequivocally as a DNase II-type enzyme. It seems that the investigated pituitary DNase represents a type of DNase which so far has not been described in mammalian tissues. It should be noted that the pituitary DNase digestion products resemble those of basic DNase from mammary tumours of C_3H mice (Georgatsos, 1966) and micrococcal exonuclease (Lehman, 1963), as in all cases only mononucleotides were found. However, other properties of these enzymes differ largely from those of pituitary DNase.

The multiple forms of DNase and RNase separated by column chromatography and electrofocusing may be due to the occurrence of isoenzymes or to proteolysis of the native enzymes to active subfractions.

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OCZYSZCZANIE I WŁAŚCIWOŚCI NUKLEAZ Z PRZYSADKI MÓZGOWEJ ŚWINI

Streszczenie

1. Otrzymano z przysadki mózgowej świni częściowo oczyszczony preparat, który zawierał aktywność dezoksyrybonukleazową i rybonukleazową.

2. Izoelektryczne frakcjonowanie preparatu dało dwie frakcje, z których każda zawierała obie aktywności.

3. Różnice we wrażliwości na podwyższoną temperaturę oraz na aktywatory i inhibitory pomiędzy aktywnością dezoksyrybonukleazową i rybonukleazową, jak również odmienna kinetyka rozkładu DNA i RNA przemawiają za tym, że aktywności te związane są z odrębnymi enzymami.

4. DNaza jest bardziej termolabilna. EDTA i jony Ca^{2+} nieco zwiększają jej aktywność, a jodooctan silnie ją hamuje. Optimum pH wynosi 5.5. W produktach częściowego i całkowitego rozkładu DNA znaleziono wyłącznie 3'-dezoksyrybonukleotydy.

5. Do aktywności RNazy nie są konieczne dwuwartościowe kationy; EDTA jest bez wpływu.

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INVESTIGATIONS OF HYDROGEN-DEUTERIUM EXCHANGE IN α-CHYMOTRYPSIN

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1. H-D exchange in *a*-chymotrypsin at pD 4.6, in the temperature range 9 - 40°C, was investigated. 2. On this basis the activation energy, and the values of enthalpy and entropy of activation were obtained. 3. These data lead to the conclusion that local segmental conformational motions are responsible for the observed rate of exchange. 4. Addition of salt ($CaCl_2$, $MgCl_2$, and NaCl) has only little influence on the rate of exchange.

Spectroscopic investigations of hydrogen-deuterium exchange of amide protons of peptide bonds in proteins can provide essential information on the influence of pH, temperature and other factors on the stability of protein conformation (Hvidt & Nielsen, 1966). In order to obtain more detailed informations on the influence of bivalent ions on the enzymic activity of trypsin and chymotrypsin (Green & Neurath, 1953; Green *et al.*, 1952) investigations were initiated on the influence of a number of salts ($CaCl_2$, $MgCl_2$, and NaCl) on the rate of hydrogen-deuterium exchange in chymotrypsin. It is well known that the influence of $CaCl_2$ on chymotrypsin activity appears at 10^{-5} - 10^{-2} molar concentrations (Jennings *et al.*, 1958), and that in a 0.023 M (0.5%) $CaCl_2$ solution each mole of this protein binds about 5 gramions of Ca^{2+} (Friedberg & Bose, 1969). The salt concentration applied in our investigations was in a similar range.

The spectroscopic method used is described in detail in literature (Blout *et al.*, 1961; Hvidt, 1963; Hvidt & Nielsen, 1966). This method assures good control of the exchange process and good reproducibility.

MATERIALS AND METHODS

Reagents. Bovine *a*-chymotrypsin (Koch-Light Lab., Colnbrook, England) was used without further purification. Calcium chloride, magnesium chloride and sodium chloride were of analytical grade (POCh., Gliwice, Poland). Deuterium oxide was of 98.3% purity (Reachim, Moscow, U.S.S.R.).

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IR-measurements. Measurements were performed on a Perkin-Elmer spectro-photometer, model 621, in the 1800 - 1300 cm^{-1} region, where a considerable transmittance for deuterium oxide is observed (Gore *et al.*, 1949). Cell thickness was 0.023 mm. A 5% protein solution in pure deuterium oxide or with addition of the appropriate salt was applied to KRS-5-plates and placed in a thermostated jacket. Temperature was controlled to $\pm 0.1°C$. As a reference D_2O or the suitable salt solution in D_2O without protein was used. A 1% solution of $CaCl_2$ (0.046 M) was used, and solutions of $MgCl_2$ and NaCl were adjusted to the same ionic strength. The first reading, in all cases, was performed about 5 min after protein solubiliza-tion. Transmittance scale was checked at 1800 cm^{-1} without cells in the beams. To determine absorption values of amide I and amide II, the tangent to the minimum absorption at 1750 cm^{-1} was adopted as the base line. As the background of amide II band (1545 cm^{-1}) at the indicated frequency the corresponding absorption value was adopted, which was measured after 3 h protein incubation at 40°C. At this temperature the exchange runs within 30 min, and further incubation during 2.5 h does not affect the spectrum. After background correction, the peak absorbance of amide II relative to amide I was assumed, according to Willumsen (1968), to be proportional to the fraction of amide hydrogens in the protein. In all measure-ments the value of amide I band was found to be constant (0.52±0.05). Salt addition caused no changes of the absorption curve and also of the value of background absorption in the frequency region of amide II band.

Measurements were performed for chymotrypsin solutions in heavy water at 9°, 20°, 30°, 35°, and 40°C; for chymotrypsin solutions in 0.046 M-calcium chloride solution in D_2O at 20°, 25°, 30°, 35°, and 40°C; for chymotrypsin solutions with magnesium chloride and sodium chloride at 20° and 35°C. For each temperature, 2 - 5 independent measurements were performed and the mean values of rate constants of exchange calculated.

The percentage of exchanged protons was determined in relation to the value $\frac{A_{amide\ II}}{A_{amide\ I}} = 0.3$, which was obtained for chymotrypsin incubated in D_2O at 9°C, 5 min after solubilization of protein (first recording of the spectrum). In all cases the percentage of exchange was calculated for time *t*, that is the end of the first stage of exchange.

pD *of medium*. This was checked by measuring directly the corresponding value for 5% protein solution in D_2O. Addition of $CaCl_2$ to 0.046 M concentration caused no change of pD value of the medium.

RESULTS AND DISCUSSION

The process of hydrogen-deuterium exchange in proteins, according to Hvidt & Nielsen (1966), is as follows:

$$N \underset{k_2}{\overset{k_1}{\rightleftarrows}} I \overset{k_3}{\longrightarrow} exchange$$
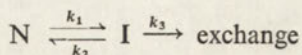
where N is the sum of all shielded protein conformations with labile hydrogen atoms, I is the sum of all conformations susceptible to exchange, k_1 and k_2 are the unfolding and refolding rate constants, respectively, and k_3 is the rate constant of hydrogen exchange in small peptides. When the protein is mainly in the N conformation, and the k_3 is considerably higher than k_2, the exchange mechanism is EX_1. In the reverse situation (k_2 considerably higher than k_3) the EX_2 mechanism is operative. In the EX_1 mechanism the rate of exchange is limited by the rate of formation of the I conformation, whereas the rate of exchange in the EX_2 mechanism is determined by the equilibrium concentration of the conformation I.

At present very little is known about the nature of conformational changes believed to be essential for protein hydrogen exchange. However, according to Hvidt & Nielsen (1966) the rate of exchange of a given, slowly exchanging peptide-group hydrogen atom in a globular protein is determined, not by the local conformational environment of the exchanging hydrogen atom in the native protein, but by the over-all folding of the polypeptide chains in the larger region of the protein molecule involved in the transconformational reaction N→I. A different point of view has been formulated by Rosenberg (Rosenberg & Chakravarti, 1968; Rosenberg & Enberg, 1969). According to this author, at low temperatures the exchange reaction is determined mainly by the segmental non-cooperative motions of the protein surface; the postulated surface mobility means that there exists a large number of conformational isomers with small differences in the folding of polypeptide chains. This is supported, according to Rosenberg (Rosenberg & Chakravarti, 1968; Rosenberg & Enberg, 1969), (who determined the activation energy of the exchange reaction for a number of proteins), by the much lower values obtained for the exchange reaction at lower temperatures in comparison with calculations and measurements carried out under conditions in which the exchange process was determined by reversible thermal denaturation. It is remarkable (Rosenberg & Enberg, 1969) that in chymotrypsinogen at pH 7 and 35°C all hydrogens exchange rapidly without involving cooperative unfolding.

The exchange in α-chymotrypsin was investigated with spectroscopic methods by Willumsen (1968) and by Bhaskar & Parker (1970). Willumsen studied the influence of pD on exchange at 21°C and observed at pD 3.1 the slowest exchange rate; he also determined that the difference between the pD-profiles for the exchange reaction in chymotrypsinogen and those in α-chymotrypsin showed no definite change during the first 2.5 h of incubation. In the opinion of Willumsen this leads to the conclusion that autolysis of the protein is not of importance in this first stage of exchange reaction. The profiles of the rate dependence on pD of the medium support the view that at least 3/4 of all peptide-group hydrogens exchange in agreement with the EX_2 mechanism. The minimum exchange rate at pH 3.5 and 22°C has been determined by Bhaskar & Parker (1970). Basing on the observed exchange rate these authors distinguish in chymotrypsin three classes of amide protons. The rate constant of exchange for the fastest exchanging protons approximately equals the rate constant of exchange in model polypeptides in random-coil conformation (k_m about 0.3 min^{-1}). In the second and third class of protons the rate

constants are about 10 and 100 times smaller, respectively. The exchange in each class of protons followed first order reaction kinetics.

In order to obtain more exact values of the activation parameters of hydrogen-deuterium exchange process in a-chymotrypsin we have followed this process over a broad temperature range, from 9 to 40°C. The measurements were performed at pD 4.6. These are conditions very close to those needed for the minimum rate of exchange in chymotrypsin.

In Fig. 1 typical changes in the IR-spectrum of chymotrypsin during incubation in D_2O are shown. The disappearance of the absorption band at 1545 cm^{-1}
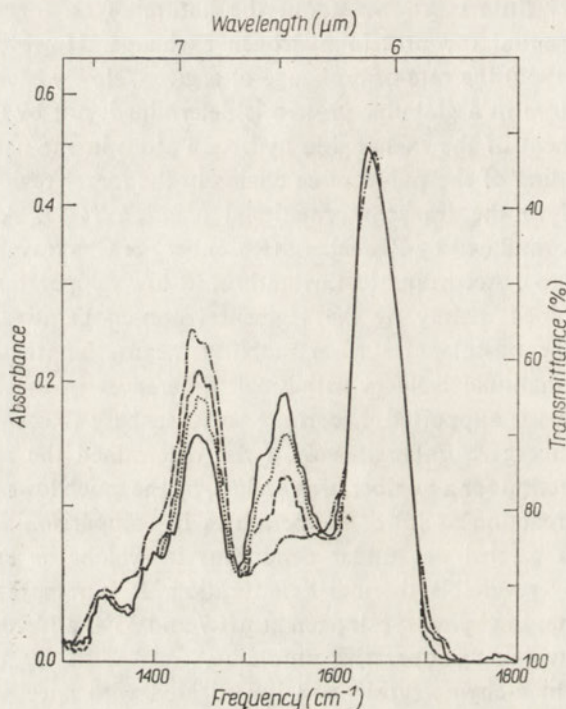
Fig. 1. Repeated scans of the infrared spectra of 5% a-chymotrypsin solution in deuterium oxide at pD 4.6, and temp. 20°C, started: 5 min (———), 50 min (·····), 420 min (— — —) after dissolution, and (·—·—·—) background spectrum for exchange of a-chymotrypsin obtained by incubation for 3 hours at 40°C in D_2O.

(NH bending vibration) is accompanied by an increase of absorption at 1450 cm^{-1} (mostly ND bending vibration). The observed changes in these regions are not always strictly parallel, because at 1450 cm^{-1} the absorption of HDO molecules is also observed (Sabato & Ottesen, 1967). Therefore traces of water in the specimen influence considerably the spectrum in this region. If the data shown in Fig. 2, which illustrate the range of exchange at the successive temperatures, are plotted on a semilogarithmic scale, then for the lower temperature (9 - 35°C) the values of

$\lg \dfrac{A_{amide\ II}}{A_{amide\ I}}$ will form two nearly straight lines. Therefore it is possible to envisage,
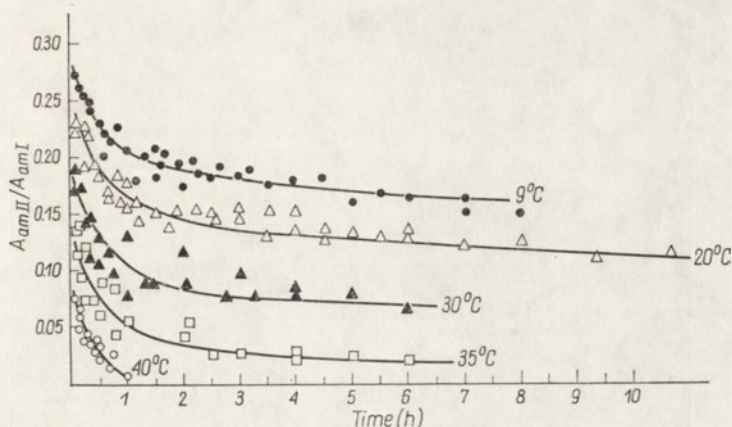
Fig. 2. The exchange of peptide hydrogens in α-chymotrypsin in D_2O at pD 4.6 represented as a function of time at the indicated temperature. In each case the data from two independent experiments were plotted on the figure.

with good approximation, the exchange process to be composed of two stages, which can be described by different rate constants. The rate constant of the first stage was determined as follows: as the starting concentration of amide protons was assumed the value which was obtained from the extrapolation of data in the semilogarithmic plot to zero time. For the second reaction stage, as the starting value of amide protons concentration was assumed the limit value of $\dfrac{A_{amide\ II}}{A_{amide\ I}}$ at time t, where a sharp change in the exchange rate was observed. Subsequently the first order rate constants of exchange were graphically determined. For the first exchange stage reproducible results were obtained with a deviation of 15% only from the mean value. The method used is illustrated in Fig. 3 for the temperature of 35°C. It may be seen that the first stage of exchange covered, similarly as at the other temperatures tested, the first 30 min of the whole process. It has to be noted here that for the second stage of exchange the experimental error increased. This was mainly due to the decreasing values of the amide II absorption band. The deviation of experimental values of the k^{II} constant, determined in independent experiments varied in some cases (mainly at 20°C) by as much as 30%. The analysis of data is further complicated at this stage by the possible denaturation of protein due to the long exposure time and enzyme autolysis. Therefore further discussion is based mainly on the results obtained for the first stage of reaction. It should be noted that, as it was mentioned above, at 40°C practically all amide protons are exchanged during the first half hour and it is not possible to divide this process into two stages. All results are summarized in Table 1.

Basing on Eyring equation (Glasstone *et al.*, 1941):

$$\lg \frac{k}{T} = 10.32 - \frac{\Delta H^*}{4.57\,T} + \frac{\Delta S^*}{4.57}$$
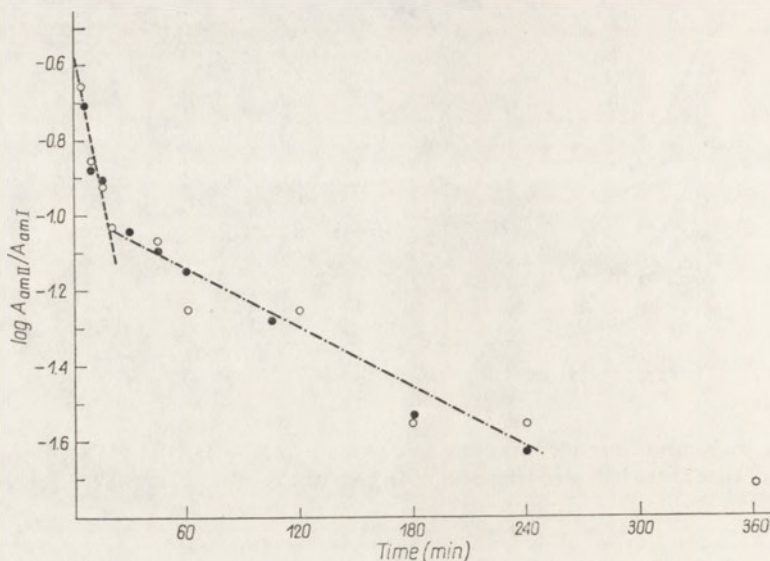
Fig. 3. The semilogarithmic plot for the rate of peptide hydrogens exchange in α-chymotrypsin in D_2O at 35°C. Points (O) and (●) represent the data from two independent experiments.

the values of enthalpy and entropy of activation of the first stage of the exchange process were determined using the least square method. Values of 12.3 kcal/mol and -32.6 cal·mol^{-1}·deg^{-1}, respectively, were obtained. The energy of activation of the reaction calculated from these values, was E* = 12.9 kcal/mol. The low value of the activation energy suggests that thermal transconformation plays really a minor role in the first stage of the exchange process. Our value is, however, in good agreement with the value of activation energy for segmental motion in ribonuclease (12 kcal) obtained by Blears & Danyluk (1967, 1968) by the pulsed magnetic resonance method. It seems, therefore, that our data support the hypothesis of Rosenberg (Rosenberg & Chakravarti, 1968) on the mechanism of hydrogen-deuterium exchange in proteins. Particularly interesting is the high negative value of activation entropy. In fact, as a result of conformational motions rendering possible the exchange, there has to be an increase in the contact area of the hydrophobic protein residues with water. This should lead to the decrease of the entropy of the whole system.

It has been observed that addition of salt to solutions of α-chymotrypsin evokes no essential changes in the shape of exchange curves. The respective rate constants calculated for both stages of exchange are summarized in Table 2. Comparing data of Table 1 and Table 2 one can see that salt addition causes only little effect on the exchange rate at both stages of the process. On the basis of experimental data, the values of enthalpy and entropy of activation for the first stage were calculated also for the salt-containing solutions. The respective values (calculated with least square method) are ΔH* = 14.7 kcal/mol, and ΔS* = -24.9 cal·mol^{-1}·deg^{-1}.

The Arrhenius energy of activation calculated from these values is 15.3 kcal/mol. These results are very close to those obtained for the first stage of exchange in absence of salt. The relatively high difference observed between the respective values of activation entropy seems to exceed, however, the possible experimental error. This may indicate that ordering of water molecules on protein surface is smaller in the presence of salts than in pure solvent.

It was mentioned above that the kinetic data obtained for the second stage of exchange have to be treated with caution. However, the decrease of exchange rate, which was consistently observed in the second stage, indicates an increase in activation energy of proton exchange at this stage. As it seems, in the first stage the mean exchange rate is determined mainly by local conformational motions. It is possible that in the second stage, clearly visible for temperatures below 40°C, other processes begin to influence the exchange reaction. These are probably connected with cooperative conformational motions of the molecule. Comparison of the respective rate constants indicates that also in the second stage of exchange addition of salt has only a minor effect.

Table 1

Exchange rate constants k^I and k^{II} for α-chymotrypsin in D_2O at pD 4.6, and percentage of hydrogens remaining at the end of the first stage of exchange

k^I is the rate constant of the first stage of exchange, k^{II} the rate constant of the second stage of exchange, and Remaining H, amide hydrogens remaining at the end of the first stage of exchange, expressed as percentage in relation to those remaining in the protein after 5 min incubation at 9°C.

Temperature (°C)	k^I (min⁻¹)	k^{II} (min⁻¹)	Remaining H (%)
9	0.0035		70
20	0.006	0.00033	60
30	0.0122	0.0014	46
35	0.0262	0.00263	30
40	0.0337		0

Table 2

Exchange rate constants k^I and k^{II} for α-chymotrypsin in 0.046 M-$CaCl_2$ in D_2O, and percentage of hydrogens remaining at the end of the first stage of exchange

Temperature (°C)	k^I (min⁻¹)	k^{II} (min⁻¹)	Remaining H (%)
20	0.0043	0.00092	47
25	0.0103	0.0011	44
30	0.0154	0.0014	30
35	0.0168	0.0022	23
40	0.0277	—	0

Table 3

Exchange rate constants k^1 *for α-chymotrypsin in the presence of* NaCl (0.138 M) *and* $MgCl_2$ (0.046 M), *and percentage of hydrogens remaining at the end of the first stage of exchange*

Salt	Temperature (°C)	k^1 (min⁻¹)	Remaining H (%)
NaCl	20	0.0088	40
	35	0.018	25
$MgCl_2$	20	0.009	40
	35	0.0178	23

It can be seen, however (Tables 1 and 2), that salt addition considerably increases the amount of exchangeable protons. The obtained value for solutions with added salt was about 15 - 20% higher than for pure solvent.

The influence of salt on the rate of exchange was mainly studied with calcium chloride, but some measurements were carried out also with magnesium chloride and sodium chloride solutions of equal ionic strength. The results presented in Table 3 indicate that the effect of these salts on the first stage of exchange is similar to that observed with calcium chloride. Thus there is no specific effect connected with a particular metal ion.

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BADANIA NAD WYMIANĄ WODÓR-DEUTER W α-CHYMOTRYPSYNIE

Streszczenie

1. Przebadano wymianę wodór-deuter w α-chymotrypsynie przy pD 4,6 w zakresie temperatur 9 - 40°C.

2. Na podstawie tych badań otrzymano energię aktywacji oraz entalpię i entropię aktywacji procesu wymiany.

3. Wyniki sugerują, że szybkość wymiany w badanym zakresie determinują lokalne ruchy konformacyjne w segmentach łańcuchów peptydowych.

4. Dodatek soli (CaCl₂, MgCl₂, NaCl) tylko w nieznacznym stopniu wpływa na szybkość wymiany.

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AN IMPROVED METHOD OF ISOLATION OF CRYSTALLINE BASIC TRYPSIN INHIBITOR FROM BOVINE TISSUES

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The elaborated improved method was applied to obtain the inhibitor from various bovine tissues. The inhibitors isolated from the thyroid gland, heart muscle and posterior lobe of the pituitary were similar to the polyvalent basic trypsin inhibitor from lung in their physico-chemical properties as well as in the spectrum of their action on proteolytic enzymes.

Bovine tissues are a rich source of the basic polyvalent trypsin inhibitor. The purified inhibitor has been obtained from the pancreas, lung, parotid gland and liver (for review see Vogel *et al.*, 1968). In our laboratory this inhibitor has been isolated in crystalline form also from the thyroid gland (Wilusz, 1971) and heart muscle (Łomako *et al.*, 1972). In the present paper an improved method for the isolation of the inhibitor is presented. The procedure has been applied to isolate the inhibitor from the tissues which are its classical sources and from tissues from which purified preparations of the inhibitor have not so far been obtained.

EXPERIMENTAL

Reagents. Bovine trypsin, 1×crystallized, and β-alanine were from Koch-Light Lab. Ltd (Colnbrook, Bucks., England); bovine chymotrypsin, 3 × crystallized, from Reanal (Budapest, Hungary); kallikrein (Padutin), containing 10 units of biological activity per ampoule, from Bayer (Leverkusen, G.F.R.); white soluble casein and acrylamide from British Drug Houses (Poole, Dorset, England); Sephadex G-25 from Pharmacia Fine Chemicals (Uppsala, Sweden); Bio-Gel P 10 from Bio-Rad Lab. (Los Angeles, Calif., U.S.A.); carboxymethylcellulose, CM-11, from Balston Ltd (Maidstone, Kent, England); *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine (TEMED) from Eastman Kodak (New York, U.S.A.); and Amido Black 10 B from Merck A.G. (Darmstadt, G.F.R.).

Inhibitor activity. One unit of anti-trypsin activity was defined as that amount which inhibited completely 1 mg of trypsin. The activity was determined according to Kunitz (1947), the enzyme being preincubated for 10 min at 35.5°C with the inhibitor in 0.1 M-phosphate buffer, pH 7.6. Where indicated, the method of Brown (1960) was used. The anti-kallikrein activity was determined as described by Brown (1960) and anti-chymotrypsin activity according to Kakade *et al.* (1970).

Protein determination. Protein was determined at 280 nm. Concentration (mg/ml) of trypsin, chymotrypsin and pure inhibitor was calculated using the following coefficients: for trypsin 0.67 (Laskowski & Laskowski, 1954); for chymotrypsin 0.495 (Wu & Laskowski, 1955), and for the inhibitor 1.2 (Wilusz, 1971). During purification of the inhibitor, the amount of protein was expressed in arbitrary units, one unit corresponding to that amount of protein which gives $E_{280}^{1 cm} = 1.00$.

Isolation of the crystalline inhibitor. Bovine tissues, fresh or frozen to -20°C were used. All steps of the procedure were carried out at room temperature. The course of inhibitor isolation is summarized in Table 1.

Table 1

Purification of the trypsin inhibitor from bovine tissues

The results were calculated per 100 g of tissue fresh weight. Total activity corresponds to milligrams of inhibited trypsin and specific activity is expressed per one $E_{280}^{1 cm}$ unit. The extinction of protein in step 1 was determined after precipitation by ammonium sulphate saturation.

Step of preparation	Lung		Pituitary posterior lobe		Thyroid		Heart	
	Activity		Activity		Activity		Activity	
	total	spec.	total	spec.	total	spec.	total.	spec.
1. HCl extract treated with NaCl	105	0.30	80	0.12	40	0.10	16	0.07
2. TCA extract of ppt. at 0.85 (NH₄)₂SO₄ sat.	73*	1.65	57*	0.69	29	2.86	9	1.11
3. (NH₄)₂SO₄ ppt. of TCA extract (dialysed)	65	1.37	53	0.66	22	2.60	8	1.06
4. CM-cellulose column chromatography	54	5.14	34	4.86	20	5.13	6.5	4.33
5. Bio-Gel P 10 chromatography	49	6.0	—	—	8	5.82	4.5	5.80
6. Crystallization	40	6.0	15.2	5.43	6.5	6.0	3.0	6.0
Yield (%)	38		19		16		19	
Purification factor		20		45		60		86

* Non-heated extract.

Step 1. The tissue was minced and extracted with mechanical stirring for 1 h with 3 vol. of 0.2 N-HCl (the thyroid gland, with 0.7 N-HCl). Then a part of inactive protein was salted out by adding NaCl *in substantia* to a final concentration of 5%, and centrifuged.

Step 2. To the obtained supernatant, ammonium sulphate was added to 0.85 saturation and the precipitated protein was extracted twice with 5% trichloroacetic

acid (TCA), the volume of each portion corresponding to 15 ml per 100 g of tissue, and the two extracts were pooled. The extracts from the thyroid and heart were heated for 15 min at 80°C and the sediment was discarded. This considerably facilitated further purification of the inhibitor. With extracts from the lung and posterior lobe of the pituitary gland, heating was not necessary.

Step 3. From the TCA extract the protein was precipitated by saturation with ammonium sulphate, dissolved in water and dialysed for 12 h against 20 vol. of 0.05 M-Na-acetate buffer, pH 4.4.

Step 4. The dialysed solution was submitted to chromatography on a CM-cellulose column equilibrated with 0.05 M-Na-acetate buffer, pH 4.4, and eluted with increasing concentration of NaCl in the same buffer. In Figs. 1 and 2 are presented the elution profiles of the inhibitor preparations corresponding, respectively, to 76 g of the pituitary posterior lobe and to 5 kg of thyroid. The inhibitor emerged in the last fraction and was well separated from inactive protein. The elution patterns of the inhibitor from other tissues were similar.
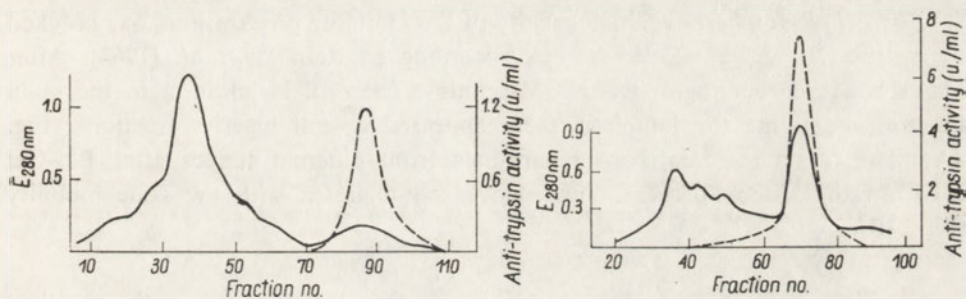

Fig. 1.   Fig. 2

Fig. 1. CM-cellulose column chromatography of protein from the posterior lobe of bovine pituitary. The protein obtained after step 3 (cf. Table 1), corresponding to 45.44 E_{280} units, was applied to the column (2×22 cm) equilibrated with 0.05 M-acetate buffer, pH 4.4. The elution was carried out with a convex NaCl concentration gradient (0 - 0.45 M). The mixing vessel contained 250 ml of 0.05 M-acetate buffer, pH 4.4, and the reservoir 0.6 M-NaCl in the same buffer. Fractions of 3.2 ml were collected at a flow rate of 30 ml/h. ——, Extinction at 280 nm; - - -, anti-trypsin units (mg of inhibited trypsin).

Fig. 2. CM-cellulose column chromatography of protein from bovine thyroid gland. The protein obtained after step 3 (cf. Table 1), corresponding to 460 E_{280} units, was applied to the column (2.5×45 cm) equilibrated with 0.05 M-acetate buffer, pH 4.4. The elution was carried out with a convex NaCl concentration gradient (0 - 0.45 M). The mixing vessel contained 1000 ml of 0.05 M-acetate buffer, pH 4.4, and the reservoir 0.6 M-NaCl in the same buffer. Fractions of 14 ml were collected at a flow rate of 90 ml/h. Other details as in Fig. 1.

Step 5. The active fractions eluted from the CM-cellulose column were pooled and the inhibitor was crystallized by adding ammonium sulphate to slight turbidity. After 24 h the first portion of the crystals was centrifuged, and to the mother liquor ammonium sulphate was again added. This procedure was repeated until the addition of the successive portion of ammonium sulphate caused sedimentation of amorphous protein. The pooled crystals were dissolved in water and purified on Bio-Gel P 10 column equilibrated with 0.01 N-HCl (Fig. 3).
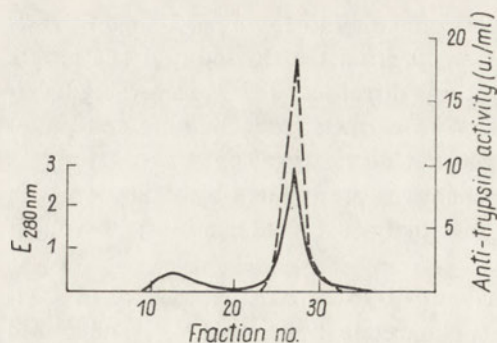
Fig. 3. Bio-Gel P 10 filtration of the crystalline trypsin inhibitor preparation from bovine heart muscle, obtained after step 4. The inhibitor solution, 4 ml (corresponding to $34 E_{280}$ units) was applied to the column $(1.5 \times 85$ cm) equilibrated with 0.01 N-HCl and eluted with the same solution. Fractions of 3.6 ml were collected at a flow rate of 25 ml/h. Other details as in Fig. 1.

Step 6. The active fractions eluted from the Bio-Gel column were pooled, adjusted to pH 4.4 with acetate buffer and the inhibitor was crystallized again as described in step 5. The obtained crystalline preparations were about 20 - 86-fold purified in comparison with the initial HCl-extract.

Disc gel electrophoresis. Homogeneity of the inhibitor preparations was checked by polyacrylamide-gel electrophoresis according to Reisfeld *et al.* (1962). After ion-exchange chromatography on CM-cellulose (step 4), in addition to the main fraction containing the inhibitor, there appeared several inactive fractions (Fig. 4A). The crystalline inhibitor preparations from different tissues after Bio-Gel P 10 filtration (step 5) were homogeneous and migrated with the same mobility (Fig. 4B).
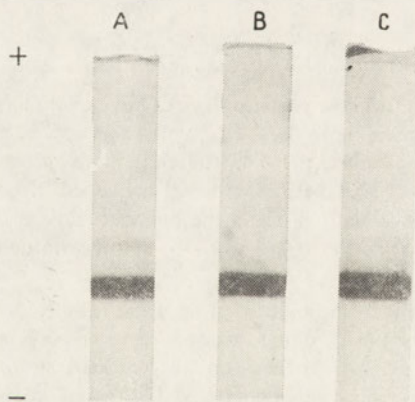
Fig. 4. Disc electrophoresis of the crystalline trypsin inhibitor preparations on 15% polyacrylamide gel at pH 4.4, according to Reisfeld *et al.* (1962), at 6 mA per tube. For separation, 30 µg of protein was applied; staining was with Amido Black. *A*, Trypsin inhibitor from posterior lobe of pituitary, crystallized after step 4; *B*, mixture of equal amounts of trypsin inhibitor preparations from lung, thyroid and heart, purified on Bio-Gel (step 5); *C*, mixture of equal amounts of the inhibitor preparations from posterior lobe of pituitary (after step 4) and from lung (after step 5).

Inhibitor activity. The homogeneous preparations of the inhibitor from lung, heart and thyroid inhibited trypsin to the same extent. One µg inhibited the activity of 5 µg of trypsin and the inhibition was linear with inhibitor concentration (Fig. 5). Similarly, each of these three inhibitors had the same effect on chymotrypsin and kallikrein; under the conditions used, 1 µg inhibited about 2.2 µg of chymotrypsin and about 1.6 units of kallikrein (Figs. 6 and 7).

The inhibitor from posterior lobe of pituitary had a somewhat smaller effect, presumably caused by the impurities not removed due to omission of the Bio-Gel filtration step.

Fig. 5   Fig. 6   Fig. 7

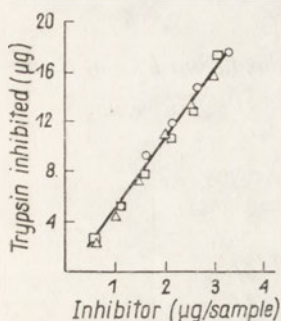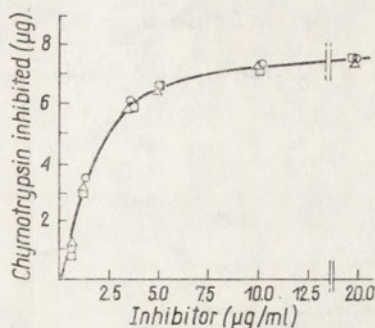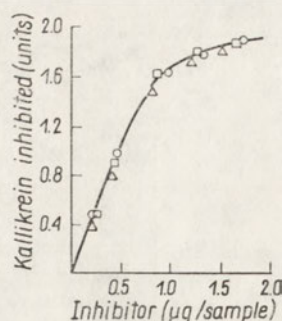Fig. 5. The effect of inhibitor concentration on trypsin activity. Conditions: 0.5 ml of the inhibitor solution in 0.2 M-tris-HCl buffer, pH 8.0, was preincubated for 10 min at 25°C with 0.5 ml of trypsin (20 µg) solution in 0.001 N-HCl - 0.02 M-CaCl$_2$; then 2 ml of a-N-benzoyl-L-arginine ethyl ester-HCl (BAEE) in 0.1 M-tris-HCl buffer, pH 8.0, containing 0.01 M-CaCl$_2$ was added. After 20 min at 25°C, 1 ml of TCA was added and the decrease of substrate was determined according to Brown (1960). Inhibitor from: ○, lung; □, thyroid gland; △, heart muscle.

Fig. 6. Effect of inhibitor concentration on the activity of chymotrypsin, determined after Kakade *et al.* (1970). The reaction mixture contained 8.4 µg of chymotrypsin, casein as substrate, and the indicated amount of the inhibitor from: ○, lung; □, thyroid gland; and △, heart muscle.

Fig. 7. Effect of inhibitor concentration on the activity of kallikrein, determined after Brown (1960). The reaction mixture contained 2 units of kallikrein, BAEE as substrate, and the indicated amount of the inhibitor from: ○, lung; □, thyroid gland; and △, heart muscle.

DISCUSSION

The method presented above permits to isolate by a simple procedure the trypsin inhibitor from various bovine tissues. In addition to preparations described in this paper, we have obtained in the same way crystalline inhibitor preparations from pancreas, kidney, spleen and rumen mucosa.

The isolated inhibitors may be classified as basic polyvalent inhibitors of proteolytic enzymes, as they exhibited a similar activity spectrum and properties as the inhibitor isolated from the lung.

As it may be seen from the data presented in Table 2, the proposed procedure permits to obtain the inhibitor in high yield and with high specific activity.

Taking into account the amount of the trypsin inhibitor extracted with dilute hydrochloric acid, bovine tissues may be divided into three groups (Table 3). To the first group belong the lung and posterior lobe of pituitary, which are very rich sources of the trypsin inhibitor. Most of the other tissues belong to the second, intermediate group. The activity of the basic inhibitor in the NaCl-treated HCl extract from pancreas could not be determined as the extract contained the acidic trypsin inhibitor, chymotrypsinogen and trypsinogen, which were active under the test conditions. The activity of basic inhibitor from 100 g of pancreas, after CM-cellulose chromatography, was 6 units. In the anterior lobe of pituitary, adrenals and brain the inhibitor was not detected. It should be noted that Brecher & Quinn

Table 2

The amount of pure crystalline preparation of the inhibitor obtained from 1 kg of tissue

One unit of the inhibitor is defined as that amount which inhibits 1 mg of trypsin.

Tissue	Inhibitor obtained (mg)	Activity (units/mg)
Lung	80	5.0
Pituitary, posterior lobe	33	4.6
Thyroid gland	13	5.0
Heart muscle	6	5.0

Table 3

Total activity of the trypsin inhibitor in the NaCl-treated HCl extract from 100 g of bovine tissues

Group	Tissue	Inhibitor activity (units)
I	Lung	105
	Pituitary, posterior lobe	80
II	Thyroid gland	40
	Kidney	34
	Spleen	28
	Heart muscle	16
	Skeletal muscle	2*
	Rumen mucosa	2
III	Adrenal gland	0
	Pituitary, anterior lobe	0
	Brain	0

* Ch. Maskos (unpublished).

(1967) reported the presence of the inhibitor bound to subcellular structure membranes in brain.

The posterior lobe of bovine pituitary gland was found to be one of the richest sources of the basic trypsin inhibitor. Therefore it seems puzzling that Lisowski & Sawaryn (1972) were unable to demonstrate its presence even by applying the method of affinity chromatography on insoluble trypsin. On the other hand, these authors claim to have isolated from this tissue an acidic protein of mol. wt. 4100, one µg of which inhibited 0.026 µg of trypsin with casein as substrate, and 0.019 µg with p-tosyl-L-arginine methyl ester as substrate. This very small inhibitory effect of the acidic protein could be due to contamination of the preparation with the basic inhibitor which, even at a concentration lower than 1%, could be responsible for the observed inhibition. The criteria of purity applied by Lisowski & Sa-

waryn (1972) are not incompatible with the presence in their preparation of a contamination of this order of magnitude. Thus it seems controversial whether the observed slight trypsin-inhibitory activity should be ascribed to the isolated acidic protein.

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OTRZYMYWANIE KRYSTALICZNEGO ZASADOWEGO INHIBITORA TRYPSYNY Z ORGANÓW BYDLĘCYCH

Streszczenie

Opisano prostą i wydajną metodę preparacji inhibitorów trypsyny z organów bydlęcych. Stwierdzono, że własności fizyko-chemiczne inhibitorów izolowanych z tarczycy, serca oraz tylnego płatu przysadki mózgowej, a także ich spektrum działania na enzymy proteolityczne, wykazują podobieństwo do zasadowego poliwalentnego inhibitora z płuc.

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COMPARATIVE STUDIES ON ORNITHINE CARBAMOYLTRANSFERASE

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1. The purified preparations of ornithine carbamoyltransferase (EC 2.1.3.3) from pea seedlings and bovine liver catalyse carbamoylation both of ornithine and putrescine; however, these two activities could not be separated on purification of both preparations. 2. The plant and liver enzymes did not differ substantially in substrate specificity and kinetic properties. 3. Ornithine carbamoyltransferase of microbial origin (fungi, bacteria) did not carbamoylate putrescine, showed higher affinity to ornithine and had quantitatively different amino acid composition as compared with the pea enzyme.

Ornithine carbamoyltransferase (OCT)[1] was isolated from bacteria (Rogers & Novelli, 1962; Bishop & Grisolia, 1967), higher plants (Kleczkowski & Cohen, 1964) and animal tissues (Reichard, 1957; Joseph *et al.*, 1963); many of these preparations were obtained in a high degree of purity (Rogers & Novelli, 1962; Kleczkowski & Cohen, 1964; Bishop & Grisolia, 1967; Zaharia & Soru, 1971). The kinetic data presented so far point to a high substrate specificity of the enzyme (Reichard, 1957; Rogers & Novelli, 1962; Kleczkowski & Cohen, 1964). However, we have recently proved that OCT of various origin is able to catalyse carbamoylation of lysine (Jasiorowska & Kleczkowski, 1970). Moreover, plant and bovine liver OCT catalyse carbamoylation of putrescine with much higher efficiency than that of lysine (Kleczkowski & Wielgat, 1968; Wielgat & Kleczkowski, 1970, 1971). This prompted us to reinvestigate substrate specificity of OCT isolated from various sources with respect to ornithine and putrescine.

MATERIALS AND METHODS

Reagents: Putrescine-2HCl (Fluka, Buchs, Switzerland); L-ornithine-HCl (Koch-Light, Colnbrook, Bucks., England); tris-(hydroxymethyl)-aminomethane and *N*-ethylmaleimide (Sigma, St. Louis, Mo., U.S.A.); L-cysteine and L-histidine

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[1] Abbreviations used: OCT, ornithine carbamoyltransferase; NC-putrescine, *N*-carbamoyl-putrescine; NEM, *N*-ethyl maleimide; pCMB, *p*-chloromercuribenzoate.

[33]

(Calbiochem, San Diego, U.S.A.); 2-mercaptoethanol (Loba-Chemie, Wien Austria); cethyl trimethyl ammonium bromide (B.D.H., Poole, Dorset, England); Sephadex G-50 fine (Pharmacia, Uppsala, Sweden); Munktel cellulose powder no. 400 for preparative electrophoresis (Grycksko Pappersbruck AB, Sweden). Carbamoyl phosphate, ammonium salt, was prepared according to Metzenberg *et al.* (1960); *N*-carbamoylputrescine-HCl and *N,N*-dicarbamoylputrescine were prepared from putrescine as described by Smith & Garraway (1964). All other reagents were from Biuro Obrotu Odczynnikami Chemicznymi (Gliwice, Poland).

Organisms. Plants were grown on moistened cotton-wool at room temperature. Bacteria were grown for 18 h at 37°C on the medium containing in 1 litre: 8 g of nutrient broth and 4 g of NaCl. *Endomycopsis vernalis* were cultivated for 44 h at 24°C on the medium containing in 1 litre: 5 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 75 g of sucrose and 10 g of urea. The sedimented cells of bacteria and *E. vernalis* were collected by centrifugation for 15 min at 6000 g. *Aspergillus nidulans* was grown for 44 h at 24°C on the basal medium described by Cove (1966).

Enzyme preparations. OCT from 10 - 14-day-old pea plants and from other sources were obtained as described previously (Kleczkowski & Wielgat, 1968). Preparations from pea and *Salmonella typhimurium* were purified up to the hydroxy-apatite column chromatography. Purification of other preparations was terminated at the heating step.

Sedimentation constants of pea and *S. typhimurium* OCT were calculated from data obtained on centrifugation of the purified enzyme in analytical centrifuge (Spinco, model E) at 52 000 r.p.m. in Na-phosphate buffer, pH 7.2. The amino acid composition of both OCT preparations was determined in automatic Beckman-Unichrome amino acid analyser, according to Spackman *et al.* (1958), after previous 24-h hydrolysis in 6 N-HCl at 105°C.

Column electrophoresis was performed using analytical LKB electrophoretic column. Purified pea OCT preparation, 50 - 100 mg, was applied to the column (30 × 400 mm) packed with Sephadex G-50 fine, or with Munktel cellulose powder. After moving the preparation with appropriate buffer to the depth of 15 cm, the flow of buffer was closed and electrophoresis was carried out for 24 h at 4°C. The following buffers were used for electrophoresis and elution: 1, 0.05 M-Na-phosphate, pH 7.2, at 30 mA/160 V; 2, the mixture of 0.9 M-tris-HCl, pH 8.6, 0.5 M-boric acid and 0.02 M-EDTA, diluted before use with 20 vol. of water to which NaCl was added to 1 mM concentration, at 18 mA/600 V; 3, the mixture of 0.02 M-Na-phosphate, pH 7.2 and 0.03 M-tris-HCl, pH 7.4, at 36 mA/240 V.

The activity of OCT was determined at 37°C as described previously (Kleczkowski & Cohen, 1964; Kleczkowski & Wielgat, 1968); the reaction products were determined colorimetrically according to Archibald (1944) and protein according to Lowry *et al.* (1951). Specific activity was expressed in μmol/1 mg protein/15 min at 37°C.

RESULTS

Specificity of OCT of various origin. According to our previous data (Kleczkowski & Wielgat, 1968), the about 3000-fold purified preparation of pea OCT catalyses synthesis of citrulline and *N*-carbamoylputrescine, the activity ratio being 10:1.

To make sure that a single catalytic protein, namely OCT, is responsible for carbamoylation of ornithine and putrescine, we attempted to separate these two activities using various procedures not included in our standard purification method. In all cases practically no change in the ratio of citrulline and *N*-carbamoylputrescine formed was observed (Tables 1 and 2). This ratio remained the same when the enzyme was extracted at pH 3.8 according to Bishop & Grisolia (1967) or was isolated by the CTAB extraction method as described by Joseph *et al.* (1963). Figure 1 presents results of column electrophoresis showing the same separation characteristics of both activities.

Table 1

Effect of pH and storage at 4°C on formation of citrulline and NC-putrescine by plant OCT, expressed as the ratio of the two activities (μmol/mg protein/15 min)

Conditions: pH 5.5, 0.05 M-succinate buffer; pH 7.2, 0.05 M-Na-phosphate buffer. Protein concentration 5 mg/ml of the appropriate buffer.

Time of storage (h)	Citrulline:NC-putrescine	
	pH 5.5	pH 7.2
0	314:31	314:31
4	250:26	280:28
24	0:0	220:23
48	0:0	200:20

Table 2

Effect of heating on formation of citrulline and NC-putrescine by plant OCT, expressed as ratio of the two activities (μmol/mg protein/15 min)

Buffers: pH 7.2, 0.05 M-Na-phosphate; pH 10.0, 0.05 M-diethanolamine (DEA) - acetic acid, or 0.05 M-glycine-NaOH. The enzymic preparation (30 mg of protein/ml) was heated in the mixture containing 1 mM-L-ornithine or 1 mM-putrescine.

1 min heating at (°C)	Citrulline:NC-putrescine		
	pH 7.2	pH 10.0 DEA	pH 10.0 glycine
61	150:15.0	30:3.5	10:1.0
71	25:2.2	0:0	0:0

Substrate specificity of OCT derived from various sources was compared with respect to ornithine and putrescine (Table 3). Out of all crude OCT preparations studied, only yeast and bacterial enzymes did not catalyse carbamoylation of putrescine. These results were confirmed with the 10 - 30-fold purified enzyme preparations.
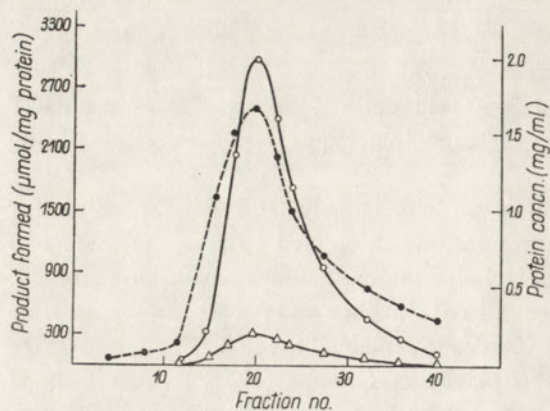
Fig. 1. Munktel-cellulose column electrophoresis of pea OCT. Formation of citrulline (○) and NC-putrescine (△). Protein concentration (●). Elution rate: 15 ml/h, fraction volume 2.5 ml.

It was also proved that the 150-fold purified preparation of *S. typhimurium* did not synthesize *N*-carbamoylputrescine in the pH range 6 - 11 even at a 200 times higher concentration of enzymic protein than that at which citrulline synthesis was observed.

Table 3

Specificity of OCT from various sources with ornithine and putrescine as substrates

Plant and bovine liver homogenates were prepared as described by Kleczkowski & Wielgat (1968). Bacteria, *Endomycopsis vernalis* and *Aspergillus nidulans* cells were disrupted in Hughes-type frozen hydraulic press block, centrifuged for 45 min at 12 000 g, and homogenates were prepared in the same medium as the plant homogenates.

Organism	Substrate	
	citrulline	NC-putrescine
	products formed (μmol/mg protein/15 min)	
E. coli	8.0	0
S. typhimurium	15.0	0
Sarcina flava	2.0	0
Endomycopsis vernalis	2.6	0
Aspergillus nidulans	2.8	traces
Pea seedlings	2.8	0.27
Barley; green seedlings	6.5	0.20
etiolated seedlings	6.0	0.20
Lupine seedlings	2.0	0.12
Mistletoe (Viscum album)	1.3	0.07
Bovine liver	8.0	0.19

Properties of OCT from S. typhimurium *and pea seedlings.* Both enzymes showed for citrulline synthesis the same pH optimum around 8.4. At pH 10.0 bacterial OCT was 6 times less active than at optimum pH, whereas the activity of pea OCT was decreased only by 30%. This implies higher sensitivity of bacterial OCT to pH changes (Table 4).

Table 4

Effect of pH *and buffer on the activity of pea and* S. typhimurium *OCT with ornithine as substrate*

Buffer and pH	Activity (μmol/mg protein/15 min)	
	pea OCT	*S. typhimurium* OCT
Diethanolamine-HCl, pH 8.4	3200	520
Diethanolamine-HCl, pH 10.0	2100	86
Propanediol-HCl, pH 10.0	2000	94
Glycine-NaOH, pH 10.0	1900	80

At pH 10.5 the plant OCT carbamoylated agmatine, spermidine and spermine but with much lower efficiency as compared with carbamoylation of putrescine (Table 5). The respective K_m values calculated for plant and *S. typhimurium* OCT are presented in Table 6. The K_m value of plant OCT for putrescine was found to be three times higher, and for agmatine five times higher, as compared with that for ornithine. K_m values for carbamoylphosphate were similar for both preparations and independent of the kind of the acceptor used. The affinity of plant enzyme to ornithine was found to be somewhat lower than that of *S. typhimurium* OCT.

Table 5

Substrate specificity of pea OCT

The purified enzyme preparation was used. The activity was determined at pH 10.5.

Substrate	Activity (μmol/mg protein/15 min)	Carbamoylation ratio amine:ornithine
Ornithine	4000	—
Putrescine	400	1:10
Agmatine	170	1:25
Spermidine	2.2	1:2000
Spermine	2.2	1:2000
Cadaverine	0	—

To gain better insight into specificity of the carbamoylation reaction, the effect of putrescine on the synthesis of citrulline by pea and *S. typhimurium* OCT was compared (Table 7). Putrescine activated synthesis of citrulline by *S. typhimurium* enzyme devoid of putrescine carbamoylation activity, in contrast to the pea enzyme which was inhibited by putrescine. This suggests that putrescine is a competitive inhibitor of plant OCT in respect to ornithine.

Table 6

K_m *values for pea and* S. typhimurium *OCT preparations*

Pea OCT was purified about 1000-fold, and *S. typhimurium* OCT about 30-fold.

Compound	S. typhimurium OCT K_m (M)	Pea seedling OCT K_m (M)
L-Ornithine	1.0×10^{-3}	2.7×10^{-3}
Carbamoylphosphate with L-ornithine	2.8×10^{-4}	2.5×10^{-4}
Carbamoylphosphate with putrescine	—	5.0×10^{-4}
Putrescine	—	8.1×10^{-3}
Agmatine	—	1.2×10^{-2}

Table 7

Effect of putrescine on the synthesis of citrulline by pea and S. typhimurium *OCT*

Putrescine (μmol/ml)	S. typhimurium OCT Activity (%)	Pea seedling OCT Activity (%)
0	100	100
2	100	100
5	108	95
10	115	90
20	130	80
40	160	40

Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Ca^{2+} ions at 0.5 - 20 mM concentration inhibited competitively carbamoylation of putrescine as it was shown with ornithine (Kleczkowski *et al.*, 1968). KCN at concentrations up to 20 mM was without any effect on the activity of pea OCT with either ornithine or putrescine as substrates. NaF inhibited completely carbamoylation both of ornithine and of putrescine at concentrations of, respectively, 10 and 5 mM. Similarly, pCMB inhibited to a higher extent carbamoylation of putrescine than that of ornithine (Fig. 2). With the OCT preparation inhibited by pCMB in 70%, L-cysteine at concentrations lower than 1 mM abolished the inhibition. At concentrations above 1 mM, L-cysteine as well as 2-mercaptoethanol, acted as inhibitors of plant, bacterial and bovine liver OCT with ornithine as substrate (Fig. 3). Iodoacetate at concentration of 5 mM inhibited in 90 - 100% the activity of *S. typhimurium* OCT, but only in 18% the pea and bovine liver OCT (Fig. 4). N-Ethylmaleimide (NEM) was found to be a strong inhibitor of plant OCT (Fig. 5) and other OCT preparations studied. Cysteine and 2-mercaptoethanol at concentrations of about 2.0 mM prevented partially the inhibitory effect of NEM and iodoacetate. L-Histidine in equimolar concentration with iodoacetate or NEM abolished this inhibition by about 50%.
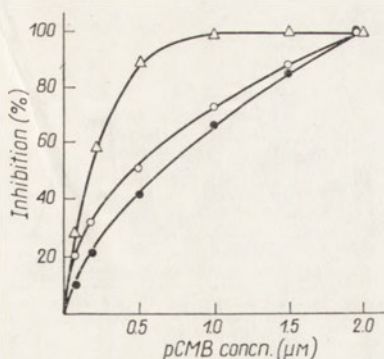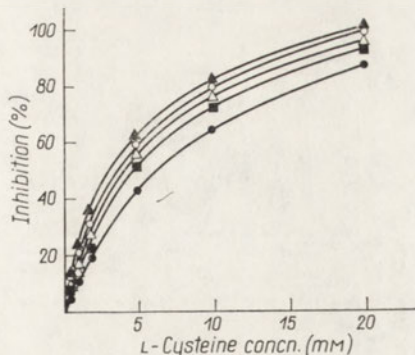
Fig. 2   Fig. 3

Fig. 2. Effect of pCMB on OCT activity. Formation of NC-putrescine ($\triangle$) by the plant enzyme, and of citrulline by plant ($\bigcirc$) and *S. typhimurium* ($\bullet$) enzymes.

Fig. 3. Effect of L-cysteine on OCT-activity. Formation of NC-putrescine by plant ($\triangle$) and bovine liver ($\blacktriangle$) enzymes, and of citrulline by plant ($\bigcirc$), bovine liver ($\blacksquare$) and *S. typhimurium* ($\bullet$) enzymes.
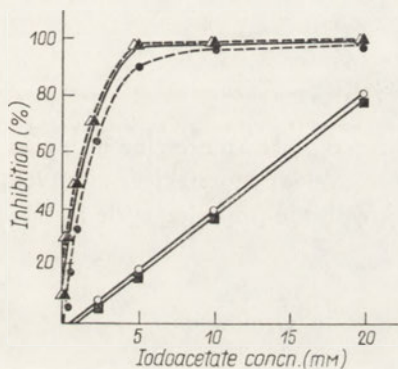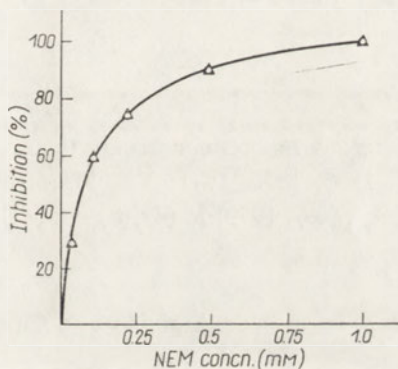
Fig. 4   Fig. 5

Fig. 4. Effect of iodoacetate on OCT activity. Symbols as in Fig. 3.

Fig. 5. Effect of NEM on the activity of OCT from pea seedlings. Formation of citrulline ($\bigcirc$) and NC-putrescine ($\triangle$).

Preincubation of the enzyme preparation with the substrates for 2 min at 37°C and pH 8.4 or 10.5, protected the enzyme against NEM and iodoacetate.

Sedimentation constants, $S_{20,w}$, after ultracentrifugation were calculated for pea OCT to be 4.80 and for *S. typhimurium* OCT, 5.33.

The amino acid composition of the purified pea and *S. typhimurium* OCT was determined using Beckman amino acid analyser. From the results presented in Fig. 6 it may be seen that the pea OCT contained more glycine, leucine, tyrosine and lysine residues but less phenylalanine and ammonia as compared with *S. typhimurium* OCT. The lack of cystine, cysteine and tryptophan residues was observed in both preparations.

Fig. 6. Amino acid composition of OCT from pea (*A*) and *S. typhimurium* (*B*).

DISCUSSION

Numerous reports concerning the occurrence of NC-putrescine in higher plants (Smith, 1965, 1970, 1971; Yoshida & Mitake, 1966; Maretzki *et al.*, 1968, 1969; Crocomo *et al.*, 1970) imply the connection with the ornithine cycle in these organisms.

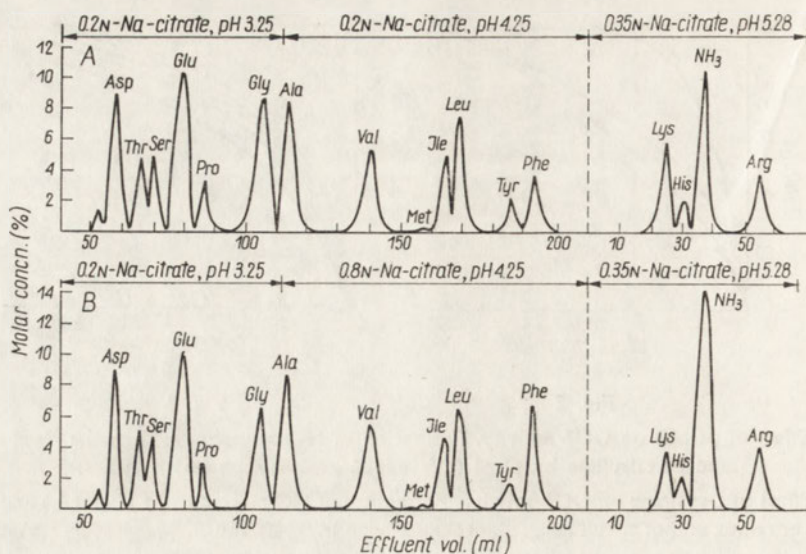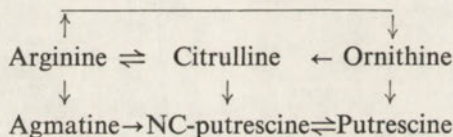$$\text{Arginine} \rightleftharpoons \text{Citrulline} \leftarrow \text{Ornithine}$$
$$\text{Agmatine} \rightarrow \text{NC-putrescine} \rightleftharpoons \text{Putrescine}$$

It has also been proved that NC-putrescine can be synthesized from putrescine and carbamoyl phosphate not only by pea OCT but by bovine liver OCT as well (Kleczkowski & Wielgat, 1968; Wielgat & Kleczkowski, 1970, 1971). The presented results prove that carbamoylation catalysed by OCT from both these sources involves also other amines such as agmatine, spermidine and spermine. In contrast, OCT of bacterial origin show absolute specificity towards ornithine. The comparative data, including K_m values, substrate specificity, optimum pH and sensitivity to SH-reagents, point to differences of OCT from various sources.

Optimum pH for all the isolated OCT preparations, with ornithine as substrate, ranged from 8.0 to 8.5 (Joseph *et al.*, 1963; Kleczkowski & Cohen, 1964; Bishop & Grisolia, 1967). However, with pea OCT the optimum pH for carbamoylation of putrescine was found to be 10.5, and at pH 8.4 only traces of activity with putrescine could be detected. Sensitivity of the enzyme to pH variation was different; the plant OCT was sensitive to low pH and more resistant to higher pH. At

pH 3.8 the enzyme from *Streptococcus* D_{10} (Bishop & Grisolia, 1967) did not lose its activity while the plant enzyme was completely inactivated. At pH 10.5 the pea OCT was only by 30% less active than at the optimum pH, in contrast to *S. typhimurium* enzyme which showed 15% of its activity at this pH.

Joseph *et al.* (1963) suggested that OCT has distinct binding sites for carbamoyl phosphate and ornithine. The same K_m values for carbamoyl phosphate independent of the origin of OCT and the substrate used, and differences in K_m values for ornithine and putrescine support this view (Table 6). Competitive inhibition by putrescine of ornithine carbamoylation by pea OCT at pH 8.4 (Table 7) suggests that ornithine and putrescine have the same binding site in the active centre: the binding efficiency for both these substrates being dependent on pH. This suggestion is also supported by identical effect of metal ions and other inhibitors on carbamoylation of putrescine and ornithine by pea OCT at pH 10.0.

The inhibitory effect of pCMB and NEM on the activity of OCT implies that SH groups are essential for enzymic catalysis. Zaharia & Soru (1971) have proved the presence of two unmasked SH groups and two S—S bridges in *Staphylococcus aureus* OCT. Grillo & Coghe (1969) suggested that SH groups are the binding sites for carbamoyl phosphate in OCT isolated from bovine liver. On the other hand, we have found neither cysteine nor cystine in pea and *S. typhimurium* OCT preparations (Fig. 6). It is known, however (Leads, 1966) that pCMB and NEM react also with an imidazole group of histidine residue, and iodoacetate with histidine, tyrosine and lysine residues. Since histidine in our experiments abolished partially the inhibitory effect of iodoacetate and NEM, this may imply that histidine residue can also be an important factor in the active centre of OCT, as it was suggested by Grillo (1970, 1971).

The differences in kinetic properties of pea and *S. typhimurium* OCT were confirmed by the quantitative differences in the amino acid profile of these two enzymic preparations (Fig. 6). These differences could be responsible for higher substrate specificity of *S. typhimurium* OCT as compared with pea OCT.

Sedimentation constants for pea and *S. typhimurium* OCT were similar and were in good agreement with the value found for rat liver OCT (Reichard, 1957). On the other hand, Bishop & Grisolia (1967) reported a higher value for *Streptococcus* D_{10} OCT, and Rogers & Novelli (1962) for *E. coli* OCT.

Data reported for molecular weight of OCT from various sources varied from 60 000 for *E. coli* OCT (Rogers & Novelli, 1962) to 360 000 for *Mycoplasma hominis* OCT (Schimke *et al.*, 1966). The values found for *S. typhimurium* and pea OCT were around 120 000 and 110 000, respectively (Wielgat & Kleczkowski, 1970), for bovine liver OCT 110 000 (Cohen & Marshall, 1962) and for rat liver OCT 156 000 (Schimke, 1962). The wide range of the reported molecular weight of OCT, from 60 000 to 360 000 depending on the origin of enzyme suggests that OCT may be composed of several subunits; it might also be that specificity of the enzyme depends on its subunit structure.

Differences found between OCT from various sources may reflect variation in the role played by these enzymes in particular organisms. For instance, besides

its main role in citrulline synthesis, plant OCT probably regulates the level of compounds which are toxic for plant cells, such as putrescine, by carbamoylating it to non-toxic NC-putrescine (Wielgat & Kleczkowski, 1971).

The results reported in this paper may also indicate that specificity of OCT is lowered in the course of evolution of living organisms.

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PORÓWNAWCZE BADANIA KARBAMOILOTRANSFERAZY ORNITYNOWEJ

Streszczenie

1. Oczyszczony preparat karbamoilotransferazy ornitynowej (EC 2.1.3.3) z siewek grochu i wątroby wołu katalizuje zarówno syntezę cytruliny jak i NC-putrescyny: rozdzielenie obu tych aktywności okazało się niemożliwe.

2. Karbamoilotransferaza ornitynowa z roślin i wątroby wołu nie wykazuje zasadniczych różnic w specyficzności substratowej i we własnościach kinetycznych. Enzym drobnoustrojów (grzyby, bakterie) charakteryzuje wyższe powinowactwo do ornityny i inny skład aminokwasowy w porównaniu z enzymem roślin.

J. TOMASZEWSKI

SOME PHYSICO-CHEMICAL PROPERTIES OF HIGH-MOLECULAR-
-WEIGHT HYPRO-PROTEIN FROM HUMAN BLOOD SERUM

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Purified hydroxyproline-containing protein (hypro-protein) from human blood
serum was found to bind specifically anti-μ antibodies. Compounds reducing the
disulphide bonds caused dissociation of hypro-protein into subunits of different
molecular weight. The amino acid and carbohydrate composition of the preparation
was determined. The obtained results indicate that the high-molecular-weight hypro-
protein contains immunoglobulin of the IgM class.

Tomaszewski (1971) described a method for purification from human blood
serum of a high-molecular-weight protein which contained more than 1% of
hydroxyproline and was immunoelectrophoretically homogeneous. In the present
work, some physical and chemical properties of this protein were studied in more
detail.

MATERIALS AND METHODS

The high-molecular-weight hydroxyproline-containing protein (hypro-protein)
was purified from sera obtained from blood donors or patients at the State Clini-
cal Hospital no. 4 in Lublin, as described by Tomaszewski (1971). Since preparative
electrophoresis resulted in considerable loss of protein, the fraction obtained by
rechromatography on Sephadex G-200 (step 4) was used.

Immunological assays. The homogeneity of the obtained protein was checked
by electrophoresis according to Scheidegger (1955) with polyvalent equine anti-
human serum (Wytwórnia Surowic i Szczepionek, Warszawa, Poland) and mono-
valent rabbit antisera against IgG, IgA, IgM and a_2-macroglobulins (Sevac, Praha,
Czechoslovakia or Behringwerke AG, Marburg-Lahn, G.F.R.). IgM was quanti-
tatively determined by radial diffusion on agar gel containing 20% of monovalent
anti-μ serum (Behringwerke) as described by Fahey & McKelvey (1965).

Reduction and alkylation. To reduce disulphide bonds, 2-mercaptoethanol
(Loba-Chemie, Vienna, Austria) was applied as described by Morris & Inman
(1970), or dithiothreitol (Calbiochem., Los Angeles, Calif., U.S.A.) as described
by Wang *et al.* (1970a). In the first case, 2.5 ml of protein solution in 0.2 M-tris
buffer, pH 7.9 (4.5 mg of protein/ml) was mixed with 1.25 ml of 0.2 M-2-mercapto-

ethanol and left for 1 h at 30°C; then the mixture was cooled to about 0°C and 1.25 ml of 0.6 M-iodoacetic acid (adjusted to pH 8) was added. After 2 h the reduced and alkylated protein was dialysed for 12 h against 0.15 M-NaCl and for 12 h against 0.2 M-tris buffer, pH 7.9, containing 0.45 M-NaCl. In the second case, 2.5 ml of the same protein solution was taken and 1.25 ml of 0.03 M-dithiothreitol was added under nitrogen. After 2 h at room temperature, the sample was cooled, submitted to alkylation with 1.25 ml of 0.6 M-iodoacetic acid as described above, then dialysed for 12 h against 0.15 M-NaCl and 12 h against 1 N-acetic acid. It is known that by the methods used only partial reduction of disulphide bonds in the oligomers of the IgM class is obtained. To achieve complete reduction of disulphide bonds, the method of Fleischman *et al.* (1963) was applied. The hypro-protein preparation was treated with 0.3 M-2-mercaptoethanol in 0.5 M-tris buffer, pH 8, for 1 h at 30°C and then alkylated.

Sephadex gel filtration. For column filtration, Sephadex G-100 (Pharmacia, Uppsala, Sweden) was used. Thin-layer gel filtration was performed on Sephadex G-200 superfine as described by Morris (1964) using as standards bovine albumin, cryst. (Light, Colnbrook, Bucks., England), trypsin, cryst. (B.D.H., Poole, Dorset, England) and normal human serum.

Polyacrylamide-gel electrophoresis. To check the purity of the isolated hypro-protein, electrophoresis on 7.5% polyacrylamide gel at pH 8.6 (Davies, 1964) was applied. Moreover, the purified protein and the reduced and alkylated preparation were submitted to polyacrylamide-gel electrophoresis in the presence of dodecyl sulphate by the method of Weber & Osborn (1969).

Analytical methods. Protein was determined according to Lowry *et al.* (1951) and in the column effluents spectrophotometrically at 280 nm. Hydroxyproline was determined by the method of Prockop & Udenfriend (1960) as modified by Tomaszewski & Hanzlik (1971).

Amino acid composition was determined (in samples containing about 2 mg of protein) after hydrolysis in 6 N-HCl, in sealed ampoules, at 110°C, for 16, 24 or 48 h. The hydrolysate was evaporated to dryness under vacuum, the residue dissolved in 4 ml of 0.2 M-acetate buffer, pH 2.2, and analysed in automatic amino acid analyser JLC - 3BC$_2$ (Japan Electron Optics Lab. Ltd) according to Spackman *et al.* (1958). The content of threonine and serine was calculated by extrapolation to zero time of hydrolysis. Cystine was assayed after oxidation to cysteic acid (Moore, 1963). Tryptophan was determined in non-hydrolysed samples as described by Opieńska-Blauth *et al.* (1963).

Hexoses were assayed by the anthrone method (Graff *et al.*, 1951) using as standard an equimolecular mixture of galactose and mannose. To determine hexosamines, protein was precipitated with ethanol and hydrolysed in 1 ml of 2 N-HCl for 4 h at 100°C; the hydrolysate was evaporated under vacuum to remove HCl, then dissolved in water, and the assay was performed according to Winzler (1955). *N*-Acetylneuraminic acid was determined according to Warren (1959) in samples hydrolysed with an equal volume of 0.1 N-H$_2$SO$_4$ for 1 h at 90°C. Fucose was determined by the method of Dische & Shettles (1948).

RESULTS

The obtained purified preparation of hydroxyproline-containing protein gave on polyacrylamide-gel electrophoresis one band near the site of application (cf Fig. 4a). On immunoelectrophoresis with antibodies against human serum proteins (Fig. 1) this preparation gave one precipitation line in the region corresponding to immunoglobulins. It bound specifically the anti-μ antibodies, whereas it did not react with monovalent antisera against IgG, IgA or α_2-macroglobulin.
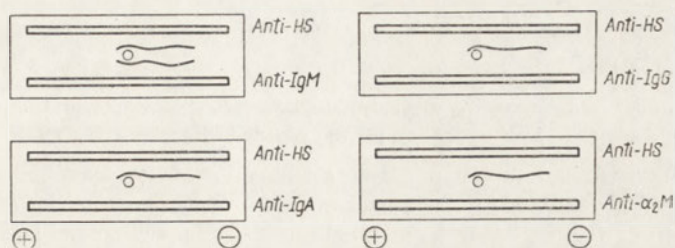
Fig. 1. Immunoelectrophoretograms of the purified high-molecular-weight hypro-protein preparation. Upper troughs, polyvalent equine serum against human serum proteins (*anti-HS*); lower troughs, rabbit antisera against human IgM, IgG, IgA and α_2-macroglobulin.

Quantitative determination of protein and determination of IgM by the radial diffusion method showed that the IgM to protein weight ratio in the hypro-protein preparation was 0.82 (Table 1).

Table 1

The content of total protein and immunoglobulins of the IgM class in the high-molecular-weight hypro-protein preparation

Protein was determined according to Lowry *et al.* (1951) and IgM by the radial diffusion method. Mean values from 6 experiments, ±S.D., are given.

Protein (mg/ml)	IgM (mg/ml)	IgM:protein weight ratio
9.4±0.3	8.1±0.7	0.82±0.07

The lyophilized hypro-protein preparation was poorly soluble in water and dilute solutions of neutral salts. The effect of pH on its solubility in 0.45 M-NaCl buffered solution is presented in Fig. 2. Storage of the protein solution in 0.45 M-NaCl or in 0.45 M-NaCl - 0.2 M-tris buffer, pH 7.9, at room temperature or at 4°C, resulted in partial sedimentation. The ratio of hydroxyproline to protein in the sediment was the same as in the starting protein. Freezing and thawing as well as lyophilization led to partial irreversible denaturation of the protein. Aggregation and formation of sediment were not prevented by addition of 6 M-urea.

The elution volume from Sephadex G-200 column equal to V_0 (Tomaszewski, 1971), and the results of thin-layer gel filtration (Fig. 3) indicate that the molecular weight of the hypro-protein corresponds to that of high-molecular-weight fraction
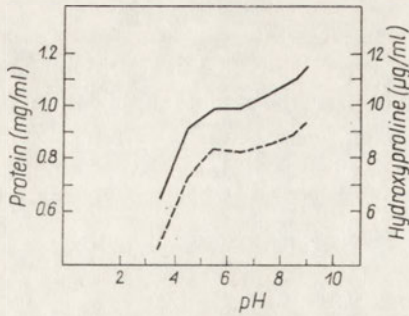
Fig. 2
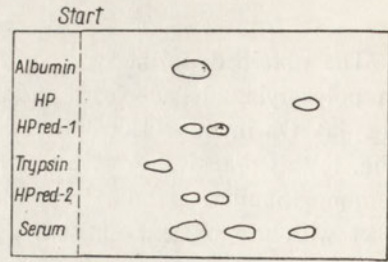
Fig. 3

Fig. 2. Effect of pH on the solubility of the lyophilized hypro-protein preparation in 0.45 M-NaCl solution. ——, Protein; - - -, hydroxyproline. pH was adjusted with 0.2 M-CH$_3$COOH or 0.2 M-NaOH.

Fig. 3. Sephadex G-200 superfine thin-layer gel filtration of the hypro-protein preparation (*HP*), of the concentrated preparations reduced with 2-mercaptoethanol (*HP red-1*) or with dithiothreitol (*HP red.-2*). As standards bovine albumin, trypsin, and normal human serum were used.

of serum protein, and is about 900 000. After reduction the spot of the high-molecular-weight protein disappeared and instead there appeared two spots of lower mobilities, corresponding to those of serum γ-globulin and albumin.

On polyacrylamide-gel electrophoresis, after partial reduction of the hypro-protein with 2-mercaptoethanol, three fractions were obtained, of which the slowest-moving one was predominant. After exhaustive reduction, on electrophoresis in the presence of sodium dodecyl sulphate the slow fraction disappeared and only the two fast-moving fractions were observed (Fig. 4).
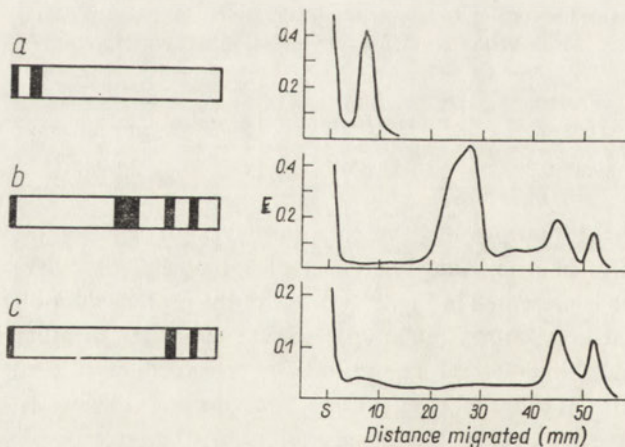
Fig. 4. Electrophoretograms on polyacrylamide gel and their densitograms. *a*, The purified hypro-protein preparation, developed in the presence of sodium dodecyl sulphate (SDS); *b*, the hypro-protein after partial reduction with 2-mercaptoethanol, separated in the absence of SDS; *c*, the hypro-protein after exhaustive reduction with 2-mercaptoethanol, developed in the presence of SDS.

On Sephadex G-100 gel filtration, the 2-mercaptoethanol-treated hypro-protein separated into three fractions (Fig. 5), two of which contained hydroxyproline. After treatment with dithiothreitol similar fractions were obtained (Fig. 6). However, the distribution of protein between the fractions was different, most of the protein being present in fraction *I*.
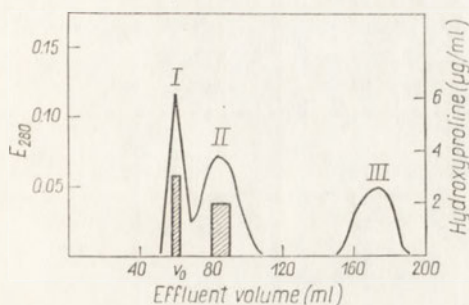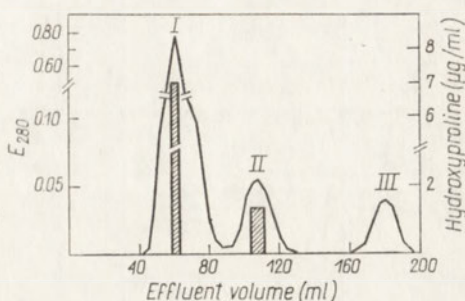
Fig. 5   Fig. 6

Fig. 5. Sephadex G-100 gel filtration of the hypro-protein preparation reduced with 2-mercaptoethanol. To the column (2.5×40 cm) equilibrated with 0.45 M-NaCl - 0.2 M-tris buffer, pH 7.9, 12 mg of protein was applied and eluted with the same buffer. In the effluent, protein was determined at 280 nm (——). The collected protein fractions were treated with trichloroacetic acid, the precipitated protein was hydrolysed with 6 N-HCl, and the content of hydroxyproline was determined (hatched columns).

Fig. 6. Sephadex G-100 gel filtration of the hypro-protein preparation reduced with dithiothreitol. To the column (2.5×40 cm) equilibrated with 1 M-acetic acid, 12 mg of protein was applied and eluted with 1 M-acetic acid. In the effluent, protein was determined at 280 nm (——). The collected protein fractions were dialysed against water, freeze-dried, hydrolysed, and the content of hydroxyproline was determined (hatched columns).

The amino acid composition of the purified hypro-protein is presented in Table 2. The acidic and neutral amino acids were predominant, aspartic and glutamic acids, threonine, serine and valine being present in the highest amounts. The content of methionine and hydroxyproline was the lowest.

The content of carbohydrates was rather high, and amounted to about 10% (Table 3). The content of *N*-acetylneuraminic acid was characteristically high. For qualitative assessment of hexosamines, the protein hydrolysed for 2 h in 2 N-HCl at 100°C was submitted to analysis in the autoanalyser; glucosamine was found to be practically the only hexosamine present in the hypro-protein.

<center>DISCUSSION</center>

It was demonstrated by immunoelectrophoresis that purified high-molecular-weight hydroxyproline-containing protein (hypro-protein) of human blood serum bound specifically the antibodies against human IgM. The comparison between the amount of protein and the amount of IgM also showed a high degree of correlation. On the basis of these results it may be assumed that serum hypro-protein contains IgM as its main or sole component.

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Table 2

The amino acid composition of the hypro-protein preparation

Amino acid	Content		
	g/100 g	residues/1000	mol/mol[a]
Lysine	4.15	37	314
Histidine	1.79	18	153
NH₃	1.47	105	892
Arginine	4.57	34	288
Aspartic acid	7.75	75	637
Threonine[b]	7.30	78	664
Serine[b]	7.45	91	774
Glutamic acid	11.65	100	850
Proline	4.85	56	476
Glycine	4.64	85	722
Alanine	3.54	52	442
Cystine (half)	1.26	15	128
Valine	6.03	66	562
Methionine	1.05	9	78
Isoleucine	2.88	28	238
Leucine	6.23	62	526
Tyrosine[b]	3.99	28	238
Phenylalanine	4.18	33	280
Tryptophan[c]	2.46	18	153
Hydroxyproline[d]	1.00	10	85
Total peptides	88.24	1000	8500

[a] Molecular weight of hypro-protein was accepted to be about 960 000.
[b] Values extrapolated to zero time hydrolysis.
[c] Determined directly according to Opieńska-Blauth *et al.* (1963).
[d] Determined by the method of Prockop & Udenfriend as described by Tomaszewski & Hanzlik (1971).

Table 3

The carbohydrate composition of the hypro-protein preparation

Component	Content	
	g/100 g of protein	mol/mol[a]
Hexose	4.90	260
Fucose	0.70	41
Hexosamines	3.20	172
Sialic acid	1.35	44
Total carbohydrates	10.15	517

[a] Molecular weight of hypro-protein was accepted to be about 960 000.

This is at variance with the conclusions of Frey (1965) and Sea *et al.* (1968). These authors, taking into account the presence in hypro-protein of hydroxyproline, the amino acid characteristic of collagen, and the presence in serum of protein which binds anticollagen antibody, suggested that the hypro-protein was an inter-

mediate in collagen metabolism. These conclusions, however, were not based on studies of purified hydroxyproline-containing protein.

In the present work a purified hypro-protein preparation was used. Its high molecular weight, corresponding to that of the macromolecular components of blood serum, and its ready aggregation resulted in high lability and low solubility *in vitro*. It may be supposed that *in vivo* solubility of the hypro-protein is favoured by the composition of the medium and the interactions of protein molecules.

Proteins of high molecular weight may be expected to be composed of subunits. Since dissociation of the hypro-protein into subunits could not be achieved either with 6 M or 8 M-urea, it may be supposed that hydrogen bonds are not essential for maintaining its quarternary structure. After reduction of disulphide bonds, the hypro-protein separated on Sephadex G-100 column into three fractions. In fraction *I* the ratio of hydroxyproline to protein was similar as in the native preparation, in fraction *II* it was significantly higher, whereas no hydroxyproline was detected in fraction *III*. The separation into three fractions suggested that reduction of the hypro-protein led to formation of two monomeric fractions (*II* and *III*), whereas a part of the oligomer remained intact. This conclusion was not confirmed by thin-layer molecular filtration on Sephadex G-200, as the spot corresponding to the high-molecular-weight hypro-protein was not found either in preparations reduced with 2-mercaptoethanol or with dithiothreitol. In both cases only two spots with mobilities corresponding approximately to those of serum γ-globulins and albumins were observed. The lack of correspondence between the number of spots and number of fractions from filtration on Sephadex G-100 could be due to smaller resolving capacity of Sephadex G-200 towards proteins of lower molecular weight, to low concentration of protein of fraction *III*, or to reaggregation of monomers into larger subunits. This last possibility seems to be the most probable in view of the results of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Under conditions of inhibited aggregation, two bands were obtained which may correspond to heavy and light monomeric subunits.

Attention should be drawn to the fact that the isolated hypro-protein is affected by the disulphide-bond reducing agents in a similar way as IgM. Depending on the conditions of IgM reduction it was possible to obtain, in different proportions, subunits IgM$_s$ (composed of four chains), monomeric heavy chains μ, and light chains (Morris & Inman, 1970; Parkhouse *et al.*, 1970; Schrohenloher, 1971), as well as different polymers of IgM$_s$ subunits (Askonas & Parkhouse, 1971). These results, together with those of the present paper, seem to be further evidence for a close relationship between the high-molecular-weight hypro-protein and IgM.

The amino acid and carbohydrate composition of the purified hypro-protein leads to similar conclusions. In Fig. 7 the amino acid composition of the hypro-protein is compared with that of IgM, as determined by Heimburger *et al.* (1964) and of α_2-macroglobulin, as determined by Demaille *et al.* (1970). In comparison with IgM, in hypro-protein only the amount of glycine was higher, by about 30%. On the other hand, from α_2-macroglobulin the hypro-protein differed in having dis-
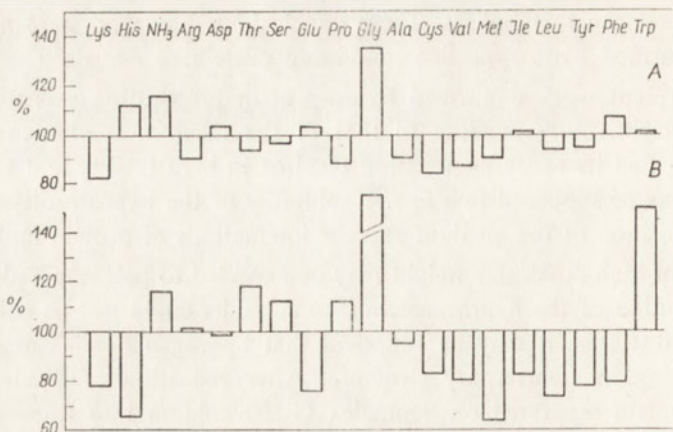
Fig. 7. A comparative diagram of amino acid composition of the purified hypro-protein preparation with: *A*, human IgM [values reported by Heimburger *et al.* (1964) being taken as 100]; and *B*, human a_2-macroglobulin [values reported by Demaille *et al.* (1970) being taken as 100].

tinctly less of histidine, glutamic acid and leucine, and more of glycine (by 67%) and tryptophan.

The hypro-protein preparation contained about 10% of carbohydrates, a value similar to that reported by Miller & Metzger (1965) for IgM, 10.22%. Recently, however, Dovie & Osterland (1968) and Miller (1970) demonstrated in studies on monoclonal protein that the content of carbohydrates in IgM may differ depending on the population of molecules. The composition of the sugar component appeared to be similar to that of IgM (Fig. 8) whereas it was different from that of a_2-macroglobulins described by Dunn & Spiro (1967), the hypro-protein containing larger amounts of hexoses, especially fucose, and smaller of hexosamines and sialic acid.

So far, the presence of hydroxyproline in IgM (Heimburger *et al.*, 1964; Suzuki & Deutsch, 1967) or in the heavy and light chains (Cohen & Milstein, 1967; Capra & Hurvitz, 1970; Habeeb *et al.*, 1970; Paul *et al.*, 1971) has not been reported.
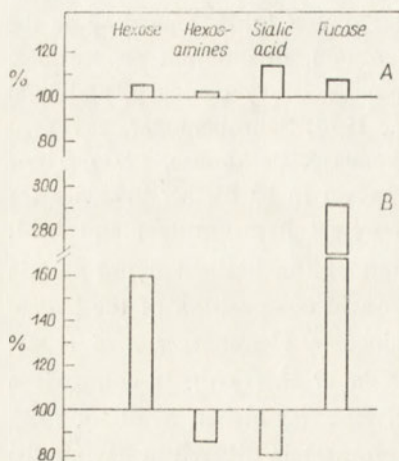

Fig. 8. A comparative diagram of carbohydrate composition of the purified hypro-protein preparation with: *A*, human IgM [mean values from data reported by Dovie & Osterland (1968), Miller & Metzger (1965) and Miller (1970) being takes as 100]; and *B*, human a_2-macroglobulin [values reported by Dunn & Spiro (1967) being taken as 100].

However, it seems possible that the rather small amounts of hydroxyproline could remain undetected by the applied methods.

The immune proteins are known to consist of a heterogeneous population of molecules differing is sequences of variable amino acid composition (Wang *et al.*, 1970b). Thus it seems highly probable that at least in some subclasses of chain μ there may exist a sequence containing hydroxyproline, or a possibility of hydroxylation of proline residues.

The mechanism of incorporation of hydroxyproline into high-molecular-weight serum proteins, its localization in the polypeptide chains and role in protein conformation require further studies. Nevertheless, the presented results, as well as those concerning the rate of turnover of the hydroxyproline-containing protein (Tomaszewski & Gilatowska, 1972) permit to suppose that the high-molecular-weight serum hypro-protein fraction contains IgM and is not related to the metabolism of tissue collagen.

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NIEKTÓRE WŁASNOŚCI FIZYKO-CHEMICZNE WYSOKOCZĄSTECZKOWEJ HYPRO-PROTEINY SUROWICY KRWI LUDZKIEJ

Streszczenie

Oczyszczony preparat białka zawierającego hydroksyprolinę (hypro-proteina) wiąże specyficznie przeciwciała anty-μ. Związki redukujące wiązania dwusiarczkowe powodują dysocjację hypro-proteiny na podjednostki o różnym ciężarze cząsteczkowym. Oznaczono skład aminokwasowy i węglowodanowy otrzymanego preparatu. Uzyskane wyniki wskazują, że wysokocząsteczkowe hypro-proteiny zawierają immunoglobuliny klasy IgM.

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PROLONGED INCUBATION OF BLOOD IN AN OPEN SYSTEM WITH BICARBONATE BUFFER

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Bicarbonate buffer and a teflon membrane permeable to CO_2 were applied for prolonged (up to 30 h) incubation of blood at a haematocrit value of 30%. Suitability of the system was assessed by measuring the pH, pCO_2, bicarbonate, Na^+, K^+, P_i, glucose utilization, haemolysis and ATP.

Prolonged blood incubation *in vitro* is indispensable for studying the interactions between the incubation medium and red cell membrane, especially for observations on lipid exchange, which is a rather slow process. It is difficult to maintain for a longer time the normal plasma environment when homeostasis of the tissue is not supported by the activity of the whole organism. First of all it is necessary to maintain the stability of pH, glucose metabolism, morphology of red blood cells, and enzymic activity of plasma. So far, prolonged (30 h) incubation of blood has not been extensively studied; this could be due to the fact that such conditions are not required for the assay of carbohydrate metabolism in red cells.

The proposed method for prolonged incubation of blood in bicarbonate buffer, providing for continuous removal through a teflon membrane of the CO_2 derived from the process of lactic acid neutralization, renders possible the maintenance of a constant pH value.

MATERIALS AND METHODS

Blood obtained from young healthy male donors was defibrinated and diluted with appropriate buffer or autologous serum to a haematocrit value of about 30% (the ratio of blood to the added buffer or serum being about 2:1). The buffers used were: bicarbonate buffer (160 mM-$NaHCO_3$ solution saturated before use with pure CO_2) and TEA buffer (160 mM-triethanolamine solution, pH 7.62). The incubation mixture contained per 1 ml: 4 mg of glucose, 2.5 mg of streptomycin and 500 units of penicillin. The volume of the incubated samples was 1.7 ml.

The incubation was carried out in 2-ml tubes of high-pressure polyethylene. The opening of the tube was covered with a teflon membrane D-602 (Radiometer, Copenhagen, Denmark) permeable to CO_2 (open system) or, for comparison, with a polyethylene cover impermeable to gases (closed system). The samples were incubated in an incubator at 37°C with continuous gentle stirring on a rotary shaker which, by continuous washing of the whole internal surface of the tube, prevented condensation of water vapour.

The samples were incubated under four sets of conditions:

A, open system with bicarbonate buffer (the proposed system), and three comparative systems:

B, closed system with bicarbonate buffer;

C, open system with autologous serum;

D, open system with TEA buffer.

In all the systems determinations were made at zero time and after 10, 20 and 30 hours of incubation.

Changes occurring in the blood during the incubation were assessed on the basis of the following analyses. The red cells count (variation coefficient 2%) and the Price-Jones plot were made using a TUR ZG-1 celloscope (VEB Transformatoren und Roentgenwerk, Dresden, G.D.R.). Haematocrit value was determined by the micro method (var coeff. 3%). Osmotic fragility of red cells was determined according to Parpart *et al.* (1947) and haemoglobin content in medium (var. coeff. 5%) according to Fleisch (1960). HCO_3^-, pCO_2 and pH were assayed using an Astrup micro apparatus type AME-1 (Radiometer) according to Siggaard-Andersen & Engel (1960). Active transport of sodium and potassium was estimated by measuring their concentration in the medium using a flame photometer (var. coeff. 2%). The glycolytic activity was estimated by determining glucose in red cells (var. coeff. 4%) by the ortho-toluidine method (Ek & Hultman, 1958, 1959). ATP in red cells was measured by the method of Adam (1965); the cells were deproteinized with 6% perchloric acid, centrifuged, the supernatant was neutralized with 1.1 M-K_2HPO_4 and kept at -20°C for determination. Inorganic phosphate (var. coeff. 4%) was determined according to Gomori (1942).

Moreover, lecithine:cholesterol acyltransferase, the enzyme catalysing esterification of cholesterol, was determined by the method of Glomset (1969), with [4-^{14}C]cholesterol (The Radiochemical Centre, Amersham, England) as substrate. The cholesterol suspension in water was submitted to sonification, then added to the external fluid of the incubated blood. The mixture was then incubated for 4 h and the ^{14}C-labelled cholesterol esters were determined using a Geiger and Müller end-window counter (PJE, AOH-42).

RESULTS

Changes in pH and bicarbonate buffer occurring during incubation of blood are presented in Table 1. System A exhibited the smallest changes in the pH value: the mean decrease during 30 h amounted to 0.21 ± 0.06. Comparison of the open

system A with the closed system B points clearly to the function of the teflon membrane. In the open systems C and D, containing only endogenous bicarbonate, the decrease of pH was greater than in system A despite the low pressure of CO_2.

Table 1

Changes in the bicarbonate buffer system during incubation of blood

A, Open system (with teflon membrane) and bicarbonate buffer; B, closed system with bicarbonate buffer; C, open system with autologous serum; D, open system with TEA buffer. The mixture, 1.7 ml, was incubated in a 2-ml tube at 37°C. The active surface of the teflon membrane was about 0.75 cm². Results of a typical experiment are given except $\varDelta$pH which represents mean values from 6 experiments, ±SD, for the decrease in pH between 0 and 30 h.

Sy-stem	Time of incubation (h)	pH	$\varDelta$pH	pCO₂ (mm Hg)	HCO₃⁻ (mEq/litre)
A	0	7.55		80.0	80.0
	10	7.49		61.0	43.0
	20	7.40		60.0	34.0
	30	7.31	0.21 ± 0.06	41.0	19.5
B	0	7.55		80.0	80.0
	10	7.14		> 150.0	> 80.0
	20	6.90		> 150.0	> 80.0
	30	6.80	0.65 ± 0.07	> 150.0	> 80.0
C	0	7.45		29.0	20.0
	10	7.21		32.0	13.0
	20	6.97		33.0	7.0
	30	6.79	0.59 ± 0.07	34.0	7.0
D	0	7.52		19.5	16.0
	10	7.44		20.0	13.0
	20	7.26		25.0	10.5
	30	7.13	0.39 ± 0.05	27.5	9.0

Glucose utilization (Table 2) was higher in system A than in the closed system B. The difference was statistically significant ($p = 0.01$). This is related to the greater decrease of pH in system B than in A. The highest ATP content in red blood cells was observed in system A, with a maximum at about 20 h of incubation. The amount of inorganic phosphate in system A increased slightly only after 30 h. The differences in the final content of inorganic phosphate between systems A and B, and A and C, after 30 h of incubation were statistically significant ($p > 0.01$).

Typical determinations of Na^+ and K^+ are presented in Table 3. Lack of changes in K^+ content in the medium in system A indicates that the activity of Na-K-dependent ATPase was maintained. In the other systems, especially in C and D, a considerable increase of K^+ ion in the medium was observed. At the same time, the increase of haemoglobin content in the medium was but slight (Table 4). This indicates that the increase in potassium was not due to haemolysis.

Table 2

Changes of glucose, ATP and inorganic phosphate during incubation of blood

Conditions as in Table 1. RBCs, red blood cells.

System	Time of incubation (h)	Glucose (mg/ml RBCs)	ATP (μmol/ml RBCs)	P_i (mEq/litre medium)
A	0	7.72	1.48	1.9
	10	4.70	2.00	2.0
	20	2.82	2.80	2.1
	30	1.68	1.10	2.6
B	0	7.65	1.48	1.9
	10	5.51	1.57	3.2
	20	3.33	1.96	5.1
	30	3.03	0.61	6.6
C	0	8.20	1.57	2.2
	10	4.90	1.62	4.7
	20	2.60	1.70	7.0
	30	2.18	0.50	8.1
D	0	7.40	—	1.2
	10	4.68	—	1.7
	20	3.25	—	2.4
	30	2.37	—	3.6

Table 3

Changes in Na^+ and K^+ in medium during incubation of blood

Conditions as in Table 1. The higher amount of Na^+ at zero time in systems A and B as compared to C is due to the presence of hyperosmotic Na-bicarbonate buffer; the lower amount of Na^+ in system D than in C is due to dilution by TEA buffer.

System	Time of incubation (h)	Na^+ (mEq/litre)	K^+ (mEq/litre)
A	0	163.0	3.0
	10	163.0	2.9
	20	166.0	3.0
	30	165.0	3.1
B	0	163.0	3.0
	10	165.0	3.1
	20	164.0	3.3
	30	166.0	4.3
C	0	142.0	4.0
	10	136.0	4.6
	20	138.0	5.7
	30	140.0	8.3
D	0	115.0	4.5
	10	117.0	7.3
	20	118.0	9.4
	30	116.0	19.2

Changes in haematocrit (Table 4) occurring in all systems studied, are consistent with the difficulties in its stabilization reported by Murphy (1967, 1968). High haematocrit value in system D at zero time and its subsequent decrease could be ascribed to the permeability of the red cell membrane to TEA, which is confirmed by the Price-Jones plot (Fig. 1). The increase in osmotic fragility of red cells and increase in haemoglobin content in medium were the smallest in system A; the content of haemoglobin in all media slightly increased. The red cells count was in all systems unchanged but it should be noted that with the applied method also the red cell membranes were counted.
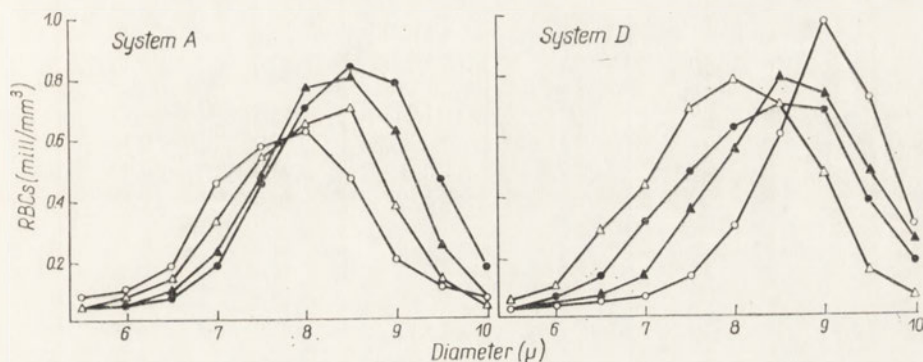

Fig. 1. Price-Jones plots for the distribution of dimensions of red cells during incubation of blood in systems A and D. Blood was diluted with the appropriate buffer by the two-stage method to a final concentration of 5×10^4. Determinations were made 30 min after the dilution. Time of incubation: ○, zero time; △, 10 h; ▲, 20 h; ●, 30 h.

The activity of lecithine:cholesterol acyltransferase (LCAT), the enzyme which is involved in the exchange of cholesterol between the red cell and serum, was determined in systems A, B and C (Table 5). No differences were observed in system A, whereas in the other two systems a decrease in LCAT activity was observed, which may be explained by greater deviation of the pH value from the optimum for enzyme activity, which decreases sharply below pH 7.5.

DISCUSSION

There are several methods for maintaining a constant pH value during incubation of blood. The simplest one consists in lowering the extent of glycolysis by diluting the blood with autologous serum to a low haematocrit value, e.g. 10% (Murphy, 1962) or 6% (Cooper & Jandl, 1969). This, however, necessitates the use of large amounts of blood both for preparation of the medium and for determinations on blood cells. Very good results have been obtained with the use of a pH-stat (Vestergaard-Bogind, 1962): carbon dioxide is removed by bubbling a mixture of nitrogen and CO_2 at appropriate partial pressure through the incubated blood. This procedure, however, permits to maintain the pH value for a period not exceeding 10 hours, and also requires the use of large volumes of blood. Addition of a buffer

Table 4

Stability of red cell membrane during incubation of blood

Conditions as in Table 1. Osmotic fragility of red blood cells is defined as that concentration of NaCl at which 50% of the cells are haemolysed.

System	Time of incubation (h)	Haematocrit (%)	Red cells count (mill/mm^3)	Hb in medium (mg/100 ml)	Red cell osmotic fragility
A	0	29	3.00	63.5	0.45
	10	30	—	64.5	0.47
	20	30	—	66.0	0.47
	30	31	3.06	75.0	0.49
B	0	29	3.02	63.5	0.45
	10	33	—	68.0	0.51
	20	33	—	93.0	0.57
	30	34	3.10	96.0	0.58
C	0	29	3.08	63.5	0.46
	10	30	—	75.0	0.50
	20	31	—	77.0	0.58
	30	31	3.12	83.0	0.58
D	0	34	3.10	56.0	0.55
	10	32	—	67.5	0.62
	20	31	—	80.5	0.64
	30	31	3.16	86.0	0.67

Table 5

Lecithine:cholesterol acyltransferase (LCAT) activity during incubation of blood

After incubation of blood for the indicated period, the medium was separated and [4-^{14}C]cholesterol was added; after 4 h the radioactivity of cholesterol ester was determined. The activity of LCAT is expressed as percentage of esterified cholesterol per 1 h.

System	Time of incubation (h)	LCAT activity
A	0	2.4
	10	2.3
	20	2.3
	30	2.4
B	0	2.4
	10	2.2
	20	2.1
	30	1.7
C	0	2.7
	10	1.9
	20	1.9
	30	1.9

to a haematocrit value of 30% may permit to prolong the incubation to more than 10 hours. However, not all buffers can be used for this purpose, for instance phosphate buffer enhances glycolysis (Rose & Warms, 1964; Vestergaard-Bogind & Scharff, 1968), and tris and TEA buffers inhibit LCAT activity (unpublished data).

The improvement proposed in the present paper consists in application of bicarbonate buffer and removal of CO_2 through a semipermeable membrane.

Comparison of the results obtained in systems A and B, which both contain bicarbonate buffer, demonstrates the role of the teflon membrane in removal of CO_2, rendering possible prolonged incubation. Comparison of systems A and D shows clearly the greater efficiency of the bicarbonate buffer over the TEA one. The carbonic acid formed from bicarbonate on neutralization of lactic acid, and its subsequent decomposition into CO_2 and water, only slightly change the pH of the medium. The amount of endogenous bicarbonate buffer present in serum (system C) is not sufficient for maintenance of constant pH over a longer time of incubation.

The proposed system is simple and may be applied to very small samples. The changes in pH of the extracellular fluid are very small, the content of Na^+ and K^+ is practically unaltered, and the haemolysis is very small. The observed increase in osmotic fragility of the red blood cells is unavoidable because esterification of cholesterol leads to a loss of free cholesterol from the red cell membranes. Changes in the pH value of about 0.2 observed under the presented conditions do not affect the displacement of red cells lipids.

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DŁUGOTRWAŁA INKUBACJA KRWI W UKŁADZIE OTWARTYM Z BUFOREM DWUWĘGLANOWYM

Streszczenie

Do długotrwałej (do 30 godzin) inkubacji krwi przy wartości hematokrytu 30% zastosowano bufor dwuwęglanowy i błonę teflonową przepuszczalną dla CO_2. Stabilność inkubowanego układu oceniano przez oznaczanie pH, pCO_2, HCO_3^-, Na^+, K^+, fosforu nieorganicznego, ATP, hemolizy oraz zużycia glukozy.

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CHANGES OF RIBOSOMAL ACTIVITY IN RAT KIDNEY AFTER UNILATERAL NEPHRECTOMY

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1. Ribosomes were isolated from normal kidney and kidney with hypertrophy induced by unilateral nephrectomy, and their activity in a cell-free system was examined. 2. At high magnesium ion concentration (22 - 25 mM), the non-enzymic binding of [^{14}C]Phe-tRNA to the ribosomes-poly U complex was twice as high in the system containing the ribosomes from hypertrophic kidney as in the control one. 3. The incorporation of phenylalanine from [^{14}C]Phe-tRNA in the presence of cytoplasmic factors from rat liver was twice as high in the system with ribosomes from hypertrophic kidney as in the control one. 4. Differences in the activity of ribosomes cannot be ascribed to differences in the activity of alkaline RNase which was found to be the same in both types of ribosomes. 5. The results suggest that during compensatory hypertrophy of the kidney the activity of ribosomes in the process of translation becomes enhanced.

Unilateral nephrectomy leads to compensatory hypertrophy of the remaining kidney accompanied by enhanced activity of various biochemical processes involved in the growth of this tissue. Within a few hours after unilateral nephrectomy, a considerable increase is observed in the incorporation of radioactive precursors into nucleic acids and protein (Halliburton, 1969; Malt, 1969). Despite the efforts undertaken in various laboratories, it has not so far been possible to determine at which step the acceleration of protein biosynthesis does occur. In our previous work (Sendecki *et al.*, 1972) we have found that unilateral nephrectomy causes an increase in the amount of heavier polysomes and more rapid incorporation of radioactive precursors into pulse-labelled RNA in the remaining kidney.

The present paper reports experiments with a cell-free system containing ribosomes from normal and hypertrophic rat kidney. The aim of the investigations was to determine whether changes observed in the rate of translation [1] were due to an alteration in the functional capacity of the ribosomes themselves to incorporate phenylalanine into polypeptides.

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[1] Preliminary communication has been presented at the X Symp. Pol. Biochem. Soc., Abstr. B-42, B-43; Poznań, 1972.

MATERIALS AND METHODS

Animals. Male rats (120 - 150 g) of the Wistar strain were maintained on standard diet and water *ad libitum.* Nephrectomy was performed as described by Sendecki *et al.* (1972). At 48 h after the operation the animals were decapitated; kidney and liver were removed to an ice-cold buffer. All further work was carried out in the cold (2 - 4°C). For each experiment 10 operated and 5 control rats were used.

Chemicals and radioactive materials. L-[U-^{14}C]Phenylalanine (sp.act. 0.1 mCi/0.088 mg) was obtained from UVVVR (Prague, Czechoslovakia). L-[U-^{14}C]Phenylalanyl-tRNA ([^{14}C]Phe-tRNA) was prepared from control liver by the method of Moldave (1963). 2,5-Diphenyloxazole (PPO) was from Packard Instrument Comp. (Dawners Grove, Ill., U.S.A.). 1,4-Di-2(5-phenyloxazolyl)benzene (POPOP) from Koch-Light Lab. Ltd (Colnbrook, Bucks., England). Polyuridylic acid (poly U) from Miles Laboratories Inc. (Elkhart, Ind., U.S.A.). Guanosine-5'-triphosphate (GTP) was purchased from Calbiochem (Lucerne, Switzerland). Millipore filters (H.A.O. 45 μ) were from Millipore Corp. (Bedford, Mass., U.S.A.). Dithioerythriol (DTE) was from Sigma Chem. Comp. (St. Louis, Mo., U.S.A.). Bentonite from Fisher Scientific Comp. (Fair Lawn, N.J., U.S.A.) was prepared according to Nicholls & Girgis (1970). Sodium deoxycholate was from Polfa (Warszawa, Poland).

Analytical methods. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. RNA, tRNA and poly U were determined spectrophotometrically at 260 nm using the specific extinction coefficient $E_{260}^{1\%} = 220$ for RNA and $E_{260}^{1\%} = 240$ for poly U.

Preparation of microsomes and ribosomes. Kidney microsomes were obtained by the method of Moldave *et al.* (1971) except that bentonite (16 mg/g wet tissue weight) was added to the buffer prior to homogenization. The sedimented microsomes were suspended in buffer and treated with sodium deoxycholate (1% final concentration) and centrifuged. The ribosomes were purified according to Moldave & Skogerson (1967), resuspended in buffer consisting of 0.25 M-sucrose, 50 mM-tris-HCl (pH 7.6), 80 mM-NH$_4$Cl, 4 mM-dithioerythriol and 25% glycerol, stored in small portions at -20°C, and used within 7 - 10 days.

Preparation of partially purified liver and kidney transferase factors. Unfractionated transferase I (aminoacyl-tRNA binding factor) and transferase II (peptidyl-tRNA translocation factor) were prepared according to Moldave *et al.* (1971) from liver and from control and hypertrophic kidney. In some cases, "pH 5 supernatant" was prepared from the post-microsomal supernatant, from which aminoacyl-tRNA synthetases, tRNAs and microsomes had been precipitated by gradual lowering of the pH value to 5.2.

Binding of [^{14}C]*Phe-tRNA to kidney ribosomes.* The non-enzymic binding of [^{14}C]Phe-tRNA to ribosomes was measured at 24°C by the method of Nirenberg & Leder (1964); details are given in the Results. [^{14}C]Phe-tRNA bound to ribosomes - poly U complex was assayed on Millipore filters, transferred to glass vials and dried. A mixture (7 ml) of 0.5% POP and 0.015% of POPOP in toluene was added and the radioactivity was measured in a Packard Tri-Carb spectrometer

or in a Polish Scintillation Counter LSB-2 with an efficiency of 80% and 50%, respectively. For each experiment, a control without poly U was assayed, and the results were corrected for the amount of [^{14}C]Phe-tRNA retained on the filters in the control sample. All values are averages of duplicate assays.

[^{14}C]*Phe-tRNA incorporation into* [^{14}C]*polyphenylalanine.* The reaction mixtures (0.25 or 0.5 ml) contained the following components: 60 mM-tris-HCl (pH 7.6), 80 mM-NH$_4$Cl, 2 mM-DTE, 0.2 mM-GTP and 8 mM-MgCl$_2$; the amount of [^{14}C]aminoacyl-tRNA, partially purified transferases, purified ribosomes and poly U is indicated in each case. After incubation at 37°C the protein synthesis was terminated by addition of 3 ml of 5% trichloroacetic acid, and after 3 - 4 h the contents of the tubes were heated in a water bath at 90 - 95°C for 10 min. The acid-insoluble material was collected on Millipore filters, washed three times with 5 ml of 5% TCA, dried and counted as described above. The incorporation is expressed as disintegrations per min per mg ribosomal protein, or as pmol of [^{14}C]phenyl-alanine incorporated per mg of ribosomal protein (500 disint./min corresponding to 1 pmol of incorporated [^{14}C]phenylalanine). The figures were corrected by subtracting zero blanks which were analogous to the proper samples but from which either ribosomes or cytoplasmic factors were omitted.

Alkaline ribonuclease activity was measured as described by Sendecki *et al.* (1972) using as standard commercial RNA (B.D.H., Poole, England) purified by the method of Kunitz (1940). One unit of RNase activity was defined as that amount of enzyme which gave the same increase in extinction as 0.0025 µg of crystalline ribonuclease (C. F. Boehringer und Soehne, GmbH, Mannheim, G.F.R.).

RESULTS

Binding of aminoacyl-tRNA to the ribosome-mRNA complex is considered as the first step in the synthesis and elongation of polypeptide chain. It has been demonstrated that this reaction may occur under different experimental conditions, depending on magnesium ion concentration in the medium. At low Mg^{2+} concentration (8 - 10 mM) aminoacyl-tRNA is bound to the ribosome-poly U complex only in the presence of GTP and a cytoplasmic aminoacyl-tRNA binding factor (transferase I), as demonstrated by Arlinghaus, Shaeffer & Schweet (1964) for reticulocyte ribosomes and by Siler & Moldave (1969) for rat liver ribosomes. On the other hand, the same authors have found that at high Mg^{2+} concentration (18 - 20 mM), the reaction between Phe-tRNA and ribosome-poly U complex is a non-enzymic process which does not depend on GTP and transferase I.

Binding of [^{14}C]*Phe-tRNA to ribosomes from normal and hypertrophic kidney.* The optimum magnesium ion concentration for the poly U-dependent non-enzymic binding of [^{14}C]Phe-tRNA to ribosomes was found to be at 22 - 25 mM (Fig. 1), and this concentration was used in further experiments. No binding was observed at Mg^{2+} concentrations lower than 5 mM. It should be noted that the optimum Mg^{2+} concentration for kidney ribosomes was somewhat higher than that obtained with liver ribosomes by Siler & Moldave (1969).

5

The saturating concentration of poly U for binding of [^{14}C]Phe-tRNA by 2.0 extinction units of control or hypertrophic kidney ribosomes was found to be 100 μg.

At the optimum concentration of magnesium ion and saturating amount of poly U, the ribosomes isolated from the hypertrophic kidney bound two to six times as much [^{14}C]Phe-tRNA as the ribosomes from control kidney, isolated and purified in the same way (Table 1). Storage of control kidney ribosomes for 3 - 4 weeks at -20°C lowered considerably their activity in the reaction of binding of aminoacyl-tRNA. On the other hand, the activity of the ribosomes from hypertrophic kidney stored for the same period under the same conditions, was not changed.
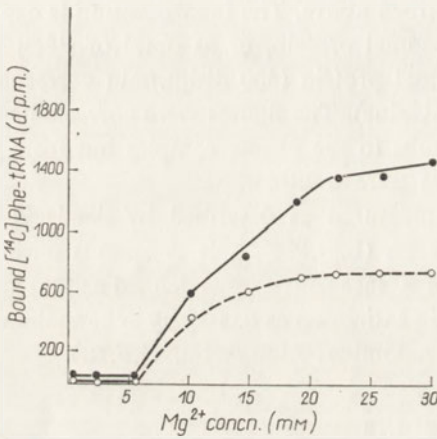

Fig. 1   Fig. 2

Fig. 1. The effect of magnesium concentration on non-enzymic binding of [^{14}C]Phe-tRNA to ribosomes from ○, control and ●, hypertrophic kidney. Ribosomes (2.0 extinction units) were incubated with 100 μg of poly U and [^{14}C]Phe-tRNA (6000 d.p.m.) in 0.25 ml of medium for 20 min at 24°C according to Nirenberg & Leder (1964).
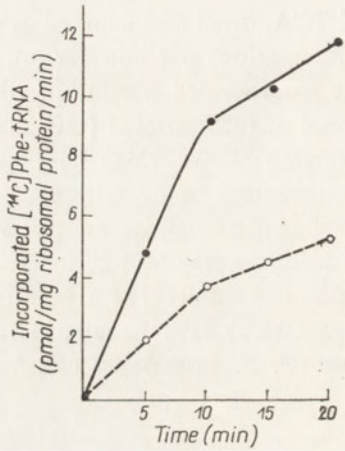
Fig. 2. Kinetics of polyphenylalanine synthesis by KCl-washed ribosome preparations from ○, control and ●, hypertrophic kidney. Ribosomes (200 μg protein) were incubated with partially purified liver transferase factors (1.5 mg of protein), 100 μg of poly U and [^{14}C]Phe-tRNA (12 000 d.p.m.) in 0.5 ml of medium, as described in Methods. Samples of 0.1 ml were withdrawn at 0, 5, 10 and 20 min and radioactivity measured as described in Methods.

Incorporation of [^{14}C]*phenylalanine from* [^{14}C]*Phe-tRNA*. Similarly as liver ribosomes, ribosomes from rat kidney washed with solutions of high ionic strength, are depleted of cytoplasmic proteins. The ribosomes which had been washed with a buffer containing 0.5 M-KCl were unable to incorporate amino acids without the addition of cytoplasmic transferase factors.

The kinetics of poly U-directed synthesis of [^{14}C]polyphenylalanine by control and hypertrophic kidney ribosomes, were studied using as cytoplasmic factors a partially purified preparation from liver. The rate of [^{14}C]phenylalanine incorporation was very rapid for the first 10 min and then declined (Fig. 2). The synthesis

Table 1

Poly U-directed non-enzymic binding of [¹⁴C]Phe-tRNA to the control and hyper-trophic kidney ribosomes

The incubation mixture contained in a total volume of 0.25 ml: 0.1 M-tris-HCl (pH 7.2), 22 mM-$MgCl_2$, 50 mM-KCl, 100 µg of polyU, [¹⁴C]Phe-tRNA (8000 d.p.m.) and 1.5 - 2.0 extinction units of control or hypertrophic kidney ribosomes. Incubation time 20 min at 24°C.

Ribosome preparation	Bound [¹⁴C]Phe-tRNA (d.p.m.)		Increasing factor
	control kidney	hypertrophic kidney	
Stored for 1 week at -20°C			
	462	834	1.8
	884	1300	1.5
	720	1410	2.0
Stored for 3 - 4 weeks at -20°C			
	210	1252	6.0
	172	1076	6.3

of polyphenylalanine was about twice as high in the system containing ribosomes from hypertrophic kidney as in the control one. The higher incorporation by the preparation from hypertrophic kidney was observed over a wide range of magnesium concentrations (Fig. 3), however, the differences were less pronounced at much
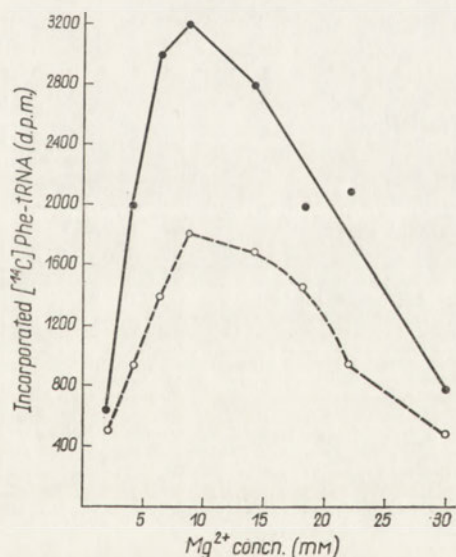

Fig. 3. The effect of magnesium concentration on the incorporation of [¹⁴C]Phe-tRNA into polyphenylalanine by KCl-washed ribosome preparations from ○, control and ●, hypertrophic kidney. Ribosomes (2.0 extinction units) were incubated with 100 µg of poly U, [¹⁴]Phe-tRNA (6000 d.p.m.) and partially purified liver cytoplasmic factors (0.5 mg of protein) in 0.25 ml of medium for 20 min at 37°C as described in Methods. The radioactivity was measured in 0.1 ml duplicate samples.

lower and much higher Mg^{2+} concentrations (4 and 18 mM). Incorporation of [^{14}C]phenylalanine from [^{14}C]Phe-tRNA directed by endogenous mRNA, which was studied under the same conditions, amounted to about 15% of the values obtained in the systems directed by poly U (Table 2) and was also higher in preparations from hypertrophic kidneys.

Table 2

Incorporation of [^{14}C]*phenylalanine from* [^{14}C]*Phe-tRNA by ribosomes from control and hypertrophic kidney in the presence and absence of poly U*

The incubation mixture (0.25 ml) composed as described in Methods, contained ribosomes (100 μg of protein), unfractionated liver transferase factors (300 μg of protein) and 100 μg of poly U. After 20 min at 37°C, two parallel 0.1 ml samples were withdrawn and radioactivity was measured.

Source of ribosomes	[^{14}C]Phenylalanine incorporated (pmol/mg ribosomal protein/min)	
	without poly U	with poly U
Control kidney	1.3	8.3
Hypertrophic kidney	3.7	21.8

Table 3

Activity of free RNase in control and hypertrophic kidney preparations

Kidney homogenates were prepared with or without bentonite, added in the amount of 16 mg/g wet kidney weight. For details see Methods.

Preparation	Prepared	
	with bentonite	without bentonite
	RNase (units/mg protein)	
Ribosomes from control kidney	0.023 (0.0 - 0.04)	2.5 (2.2 - 2.8)
Ribosomes from hypertrophic kidney	0.026 (0.0 - 0.05)	2.3 (2.0 - 2.6)
Cytoplasmic fraction from control kidney	2.85 (2.8 - 2.9)	17.1 (16 - 18)
Cytoplasmic fraction from hypertrophic kidney	3.3 (3.2 - 3.5)	17.8 (16.1 - 19.5)

Since the kidney is known to have a high ribonuclease activity (Roth, 1956), we have checked whether the observed differences in incorporation and binding ability between the normal and hypertrophic kidney did not result from the changes in the activity of this enzyme. The data presented in Table 3 show that in kidney the activity of ribonuclease was practically the same in both types of ribosomes, as well as in both cytoplasmic fractions.

DISCUSSION

Only a few investigations have been carried out on the cell-free system for protein synthesis isolated from kidney. Nicholls *et al.* (1970) and Girgis & Nicholls (1971) reported that the factor(s) responsible for the increase in protein synthesis in nephrotic kidney are present both in the cytoplasm and in ribosomes. Trachewsky *et al.* (1972) described changes in the activity of rat kidney ribosomes in the process of translation after administration of aldosterone and other steroids.

Halliburton, who studied the process of translation in the hypertrophic kidney, found no differences in the incorporation of radioactive amino acids between the systems containing microsomes from hypertrophic and normal kidneys. At the same time he found that incorporation *in vitro* of [^{14}C]leucine by both kinds of kidney ribosomes was several-fold lower than in an analogous system isolated from liver (see review by Halliburton, 1969).

Our results indicate that the ribosomes from the kidney undergoing compensatory hypertrophy incorporate much more of [^{14}C]phenylalanine than do the ribosomes from control kidney. On the average the incorporation was twice as large. Also the binding of [^{14}C]phenylalanyl-tRNA to ribosomes-poly U complex was much higher in the system containing the ribosomes from kidneys of animals which had been subjected to unilateral nephrectomy.

As the differences between the two types of ribosomes were observed also in the system incorporating [^{14}C]phenylalanine from [^{14}C]Phe-tRNA in the presence of liver transferases, it seems that the factors responsible for increased binding of aminoacyl-tRNA to ribosomes, as well as for the increased incorporation, are of ribosomal origin. This does not, of course, exclude the existence in the hypertrophic kidney of factors stimulating protein synthesis, independent of ribosomes. We have found (W. Sendecki, J. Patzer, M. Kuliszewski & M. Ombach, in preparation) that in a system containing liver ribosomes and kidney supernatant binding factors, the incorporation of radioactive amino acids was higher in the presence of factors from the hypertrophic kidney than in the control system. Similar ribonuclease activity found in both types of ribosomes indicates that neither increased binding of [^{14}C]Phe-tRNA, nor increased incorporation may be dependent on possible changes in the activity of this enzyme (Table 3). As in our experiments we have used [^{14}C]Phe-tRNA, this increase cannot be dependent, either, on increased activity of phenylalanyl-tRNA synthetase.

Thus it seems that during compensatory hypertrophy of the kidney, in addition to increased synthesis of mRNA and quantitative and qualitative changes in the polysome fraction (Sendecki *et al.*, 1972), the activity of the ribosomes themselves becomes altered. Several authors have described changes in the activity of ribosomes and microsomes in cell-free protein-synthesizing systems. Von der Decken & Hultin (1958) described the increased incorporation of amino acids into protein by microsomes from regenerating liver. Changes in the activity of the ribosomes may be also the result of hormonal influence, e.g. administration of 17β-oestradiol to ovariectomized rats caused an increase in the amount of polysomes in the uterus.

These new-formed polysomes showed a higher activity in incorporation of amino acids than the polysomes from uterus of control animals (Teng & Hamilton, 1967). Wool & Cavicchi (1967) and Castles *et al.* (1971) reported that ribosomes from muscles of rats with experimental diabetes were less active in the incorporation of [^{14}C]phenylalanine, whereas in the presence of high magnesium ion concentration (12.5 - 20 mM) they bound more of [^{14}C]Phe-tRNA.

Our results permit to suppose that during compensatory hypertrophy of the kidney following unilateral nephrectomy, ribosomes undergo some changes leading to their enhanced activity in the process of translation. That in the ribosomes of hypertrophic kidney some structural alteration may have occurred, is suggested by the experiments showing that control ribosomes stored at -20°C for 3 - 4 weeks lost a part of their activity for binding of aminoacyl-tRNA (Table 1), in contrast to ribosomes from hypertrophic kidney, purified and stored under the same conditions.

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ZMIANY AKTYWNOŚCI RYBOSOMÓW W NERCE SZCZURA PO JEDNOSTRONNEJ NEFREKTOMII

Streszczenie

1. Porównywano aktywność rybosomów nerek normalnych i przerastających w wyniku jednostronnej nefrektomii, do wiązania [^{14}C]fenyloalanylo-tRNA oraz do syntezy polifenyloalaniny w układach bezkomórkowych.

2. Przy wysokim stężeniu jonów magnezu (22 - 25 мм) nieenzymatyczne wiązanie [^{14}C]Phe-tRNA do kompleksu rybosomy-poli U było dwukrotnie wyższe w układzie zawierającym rybosomy z nerek przerastających.

3. W syntetyzujących polifenyloalaninę układach zależnych od czynników cytoplazmatycznych z wątroby szczura, rybosomy z nerek w czasie kompensacyjnego przerostu były dwukrotnie bardziej aktywne niż rybosomy z nerek kontrolnych.

4. Aktywność alkalicznej RNazy w obu typach rybosomów była jednakowa, wzrost aktywności rybosomów w omawianych układach nie może więc być zależny od zmiany aktywności tego enzymu.

5. Wyniki te sugerują, że w czasie kompensacyjnego przerostu nerki następuje wzrost aktywności rybosomów w procesach translacji.

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DIRECT SYNTHESIS OF THE 2'-O-METHYL ANALOGUES OF 1-β-(D-ARABINOFURANOSYL) CYTOSINE AND URACIL AND SOME RELATED DERIVATIVES

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1. An unambiguous synthesis of 1-β-(2-O-methyl-D-arabinofuranosyl)cytosine (2'-O-methyl-ara-C) is described, based on dimethylsulphate treatment in aqueous alkaline medium of the 3',5'-di-O-tetrahydropyranyl derivative of ara-C, followed by acid hydrolysis of the pyranyl protecting groups. The corresponding 2'-O-methyl-ara-U was then obtained by bisulfite deamination of ara-C. 2. Under the reaction conditions employed, significant methylation of the cytosine amino group occurred, and this was profited from to isolate the N^4-methyl and the N^4,N^4-dimethyl derivatives of 2'-O-methyl-ara-C. In addition, and in sharp contrast to previous findings with cytidine, about 25% of the overall product of methylation of ara-C in alkaline medium was methylated on the cytosine ring N_3 and, as a result of the instability in alkaline medium of 1,3-disubstituted cytosines, led to the appearance amongst the reaction products of 2'-O,N_3-dimethyl-ara-U, which was also isolated.

The widespread interest in ara-C[1] stems largely from its antimetabolic activity (Cohen, 1966), including antiviral (Prusoff, 1967) and anti-tumour (Sartorelli & Creasy, 1968; Roy-Burman, 1970) properties. This has prompted the synthesis of a number of ara-C analogues containing a modified aglycone (Fox, Miller & Wempen, 1966; Wempen, Miller, Falko & Fox, 1968). Less attention has been directed to modifications of the carbohydrate moiety, e.g. by alkylation of one or more of the sugar hydroxyls. Gish, Kelly, Camiener & Wechter (1971) and Montgomery & Thomas (1972) recently reported the preparation of a series of 5'-O-acyl derivatives of ara-C, some of which were shown to exhibit marked superiority as immunosuppressive and antileukemic agents as compared to the parent ara-C.

We have elsewhere shown that the relative resistance to alkylation in strongly alkaline medium of the ring N_3 of 1-substituted cytosines (Kuśmierek & Shugar, 1971) could be profited from to alkylate the various sugar hydroxyls of cytosine

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[1] Abbreviations used in this text: ara-C, 1-β-(D-arabinofuranosyl)cytosine; ara-U, 1-β-(D-arabinofuranosyl)uracil.

nucleosides, including ara-C; subsequent column chromatography made possible the separation of all three mono-O'-methyl, the three di-O'-methyl and the tri-O'-methyl derivatives (Darżynkiewicz, Kuśmierek & Shugar, 1972). Simultaneously we described a direct synthesis, from the known 5'-O-methylcytidine (Kuśmierek & Shugar, 1971), of 5'-O-methyl-ara-C (Giziewicz, Kuśmierek & Shugar, 1972), which served as a reference compound for identification of the products of mono-alkylation of ara-C (Darżynkiewicz et al., 1972). Deamination with either bisulfite (Shapiro, Servis & Welcher, 1970; Hayatsu, Wataya & Kai, 1970) or acetic acid (Notari, Lue Chin & Cardoni, 1970; Giziewicz et al., 1972) gave the corresponding 5'-O-methyl-ara-U analogue.

In a continuation of efforts to develop direct and unambiguous synthetic routes to the various O'-alkylated ara-C and ara-U analogues, we have found that 2'-O-methyl-ara-C may be prepared by an extension of the procedure described by Kanai, Kojima, Maruyama & Ichono (1970) for the synthesis of ara-C from cytidine via the 2,2'-anhydro derivative. The procedure employed led also to the isolation of several additional new ara-C and ara-U analogues, while the overall results throw some additional light on the mechanism of ring and sugar alkylation of cytosine nucleosides in alkaline medium.

RESULTS AND DISCUSSION

Specific methylation of the 2'-OH of ara-C requires the use of protecting groups for the 3'- and 5'-hydroxyls, stable in alkali and acid labile. It was assumed at the start that such protecting groups could most simply be introduced directly on 2,2'-anhydro-ara-C (I, Scheme 1), in which the 2'-OH is blocked, but can be readily released by mild alkaline hydrolysis.

Attempts to tritylate I were abandoned because of the low solubility of the latter in pyridine, as well as the insolubility in alkaline aqueous medium of 3',5'-di-O-trityl-ara-C. It should be noted, nonetheless, that trityl protecting groups have been employed in the preparation of O'-methyl nucleosides with the aid of methyl iodide (Furukawa, Kobayashi, Kanai & Honjo, 1965; Codington, Cushley & Fox, 1968; Blank, Frahne, Myles & Pfleiderer, 1970). Furthermore, mono-tritylated nucleosides are sufficiently soluble in alkaline aqueous dimethoxyethane to permit of alkylation of the sugar hydroxyls with dimethyl or diethyl sulphate (Kuśmierek & Shugar, 1971; Kuśmierek, Giziewicz & Shugar, 1973).

Attention was then directed to the use of tetrahydropyranyl protecting groups, known to be stable to alkali, and sufficiently acid labile (Smith, Rammler, Goldberg & Khorana, 1962). Attempts to pyranylate I with the use of dioxane as solvent according to Griffin, Jarman & Reese (1968), were unsuccessful due to the low solubility of I. However, dimethylformamide was eventually found suitable for this purpose, and treatment of I with 2,3-dihydropyran in the presence of p-toluenosulfonic acid at room temperature, followed by addition to the reaction mixture of sodium methoxylate to hydrolyse the 2,2' linkage, gave 3',5'-di-O-tetrahydropyranyl-ara-C (II), which was isolated by chloroform extraction. The final product
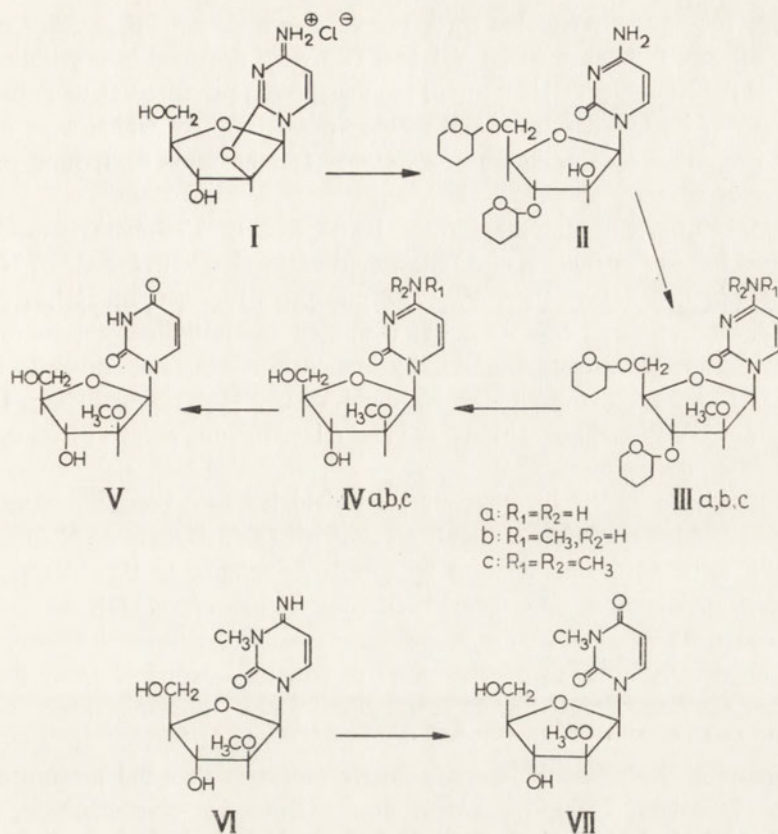
Scheme 1

was shown by TLC on HF_{254} silica gel with solvent *A1* (see Table 1) to consist of a mixture of stereoisomers in the ratio 1:10, of which the predominant component crystallized out slowly from ethyl acetate.

Treatment of II in aqueous alkaline medium with dimethyl sulphate gave 2′-O-methyl-3′,5′-di-O-tetrahydropyranyl-ara-C (III) as the major product. Hydrolysis of this in 20% CH_3COOH yielded 2′-O-methyl-ara-C (IVa), which was crystallized from anhydrous ethanol in the form of the HCl salt.

Bearing in mind the previous observation that alkylation of 1-substituted cytosines in strongly alkaline medium leads to partial alkylation of the exogenous amino group, an examination was made of the mother liquors following crystallization of IVa. TLC on HF_{254} silica gel with solvent *A2* revealed the presence of four spots, identified as follows:

    1. 2′-O-methyl-ara-C (IVa), $R_F = 0.11$
    2. 2′-O,N^4-dimethyl-ara-C (IVb), $R_F = 0.29$
    3. 2′-O,N^4,N^4-trimethyl-ara-C (IVc), $R_F = 0.37$
    4. 2′-O,N_3-dimethyl-ara-U (VII), $R_F = 0.54$

These were subsequently isolated on a preparative scale on PF_{254} silica gel with the same solvent, following which VII and IVb were obtained in crystalline form, the latter as the HCl salt. The identity of the unexpected product VII was established on the basis of its UV spectrum and melting point, both of which were identical with those reported by Codington *et al.* (1968) for the same compound prepared by a different route.

The apparently chromatographically homogeneous 2'-*O*-methyl-ara-C (IVa) *isolated from the mother liquors*, and following deamination with the aid of $NaHSO_3$ (Shapiro *et al.*, 1970; Hayatsu *et al.*, 1970), gave two products on HF_{254} gel with solvent *A2*. One of these, $R_F = 0.42$, was identified spectrally and chromatographically (Table 1) as 2'-*O*-methyl-ara-U, by comparison with an authentic sample. The other, $R_F = 0.54$, proved to be identical with 2'-*O*,N_3-dimethyl-ara-U (VII) on the basis of UV spectrum, melting point and mixed melting point with an authentic sample.

The appearance of VII in relatively high yield led to a reexamination of our previous observation that, even at highly alkaline pH (~ 13.5), the ring N_3 of cytidine is not fully resistant to methylation by dimethylsulphate. In 0.5 N-KOH, about 5% of 3-methylcytidine was formed (Kuśmierek *et al.*, 1973). In addition, with both solvents *A1* and *A2*, the mobilities of 1-substituted and 1,3-disubstituted cytosines apparently differ to only a minor extent, thus explaining why the 2'-*O*-methyl-ara-C isolated from the mother liquors, above, was mistakenly assumed to be homogeneous. The appearance, following deamination, of an appreciable proportion of 2'-*O*,N_3-dimethyl-ara-U, clearly indicates that the presumed 2'-*O*-methyl-ara-C, isolated from the mother liquors following crystallization, in fact consisted of a mixture of 2'-*O*-methyl-ara-C and 2'-*O*,N_3-dimethyl-ara-C (VI) in the ratio 5:2, as determined by elution and spectrophotometry.

The presence of 2'-*O*,N_3-dimethyl-ara-C in the products of methylation of II with dimethyl sulphate in alkaline medium may be accounted for by either of the following two pathways (Scheme 2):

(a) By deamination of the cytosine ring, followed by rapid methylation of the ring N_3 of the resulting uracil derivative; or,

(b) By methylation of the cytosine ring N_3, followed by deamination under the reaction conditions employed.

It should likewise be recalled that 1,3-disubstituted uracils readily undergo decomposition in alkali (Shugar & Fox, 1952; Janion & Shugar, 1960). Consequently the amount of 2'-*O*,N_3-dimethyl-ara-U actually found will be determined by the difference between its rate of formation and decomposition.

Pathway (a) appears an unlikely candidate in that it would not account for the appreciable proportion ($>25\%$) of VII relative to the major products formed. Alkaline hydrolysis of RNA, widely employed for isolation and quantitative estimation of mononucleotides, is usually conducted at OH^- concentrations of 0.3 N to 1.0 N for periods up to 24 hours at room temperature or at 37°C. It has long been known that, under these conditions, deamination of cytidine-2'(3')-phosphate proceeds, at most, to the extent of 1 - 2%. This extremely low rate of deamination
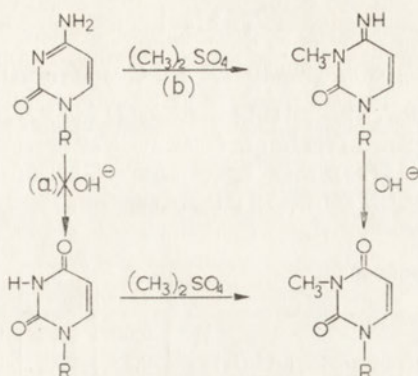
Scheme 2

is well below the rate of ring N_3 methylation of uridine and the rate of alkali-catalysed decomposition of N_3-methyluridine (Szer & Shugar, 1960).

By contrast, pathway (b) is fully supported by the relatively rapid rate of deamination of 1,3-disubstituted cytosines in alkaline medium (Brookes & Lawley, 1962).

UV spectral data for the various compounds are presented in the Experimental section. Of particular interest are the spectral changes in strongly alkaline medium (pH 12 - 14), where the sugar hydroxyls undergo ionization (Fox & Shugar, 1952). These will be reported elsewhere, together with the corresponding spectra for other O'-methyl derivatives of ara-C and ara-U, and the inferences which may be drawn therefrom regarding the acidities of the various hydroxyls of arabinosyl nucleosides, as well as the conformations of the nucleosides themselves.

Somewhat puzzling is the high degree of methylation undergone by the cytosine ring N_3 of ara-C (about 25%) as compared to that observed in the case of cytidine under analogous conditions (about 5%). Furthermore, mild methylation of ara-C with dimethyl sulphate in 1 N-KOH apparently proceeded without formation of products methylated on the ring N_3 (Darżynkiewicz et al., 1972). This problem is being subjected to further study. It is, however, clear from the present findings that the extent of ring N_3 methylation cannot be estimated quantitatively from the isolated products and that the mother liquors, following crystallization, must also be analysed.

EXPERIMENTAL

Melting points, uncorrected, were determined on a Boetius microscope hot stage. Paper chromatography was on Whatman paper no. 1. Thin-layer chromatography made use of Merck silica gel HF_{254} for analytical purposes, and PF_{254} silica gel for preparative separations. Solvent systems are listed in Table 1, which presents also R_F values on HF_{254}, but these differed only minimally, if at all, from the corresponding R_F values on PF_{254}. Ultraviolet absorption spectra were run on a Zeiss (Jena) VSU-2P instrument, and also on a Perkin-Elmer Model 450 recording spectrophotometer.

Table 1

Ascending chromatography of ara-C and ara-U derivatives

Chromatography was on HF_{254} silica gel (with solvents *A1* and *A2*) and on Whatman paper no. 1 (with solvents *B*, *C* and *D*). Solvent systems as follows with all proportions expressed as v/v: (*A1*): chloroform - methanol, 85:15; (*A2*): chloroform - methanol, 80:20; (*B*) n-butanol - water, 84:16; (*C*) isopropanol - conc. NH_4OH - H_2O, 7:1:2; (*D*) n-butanol - glacial acetic acid - H_2O, 5:2:3.

Compound	R_F in solvent system				
	A1	A2	B	C	D
Ara-C	0.00	0.05	0.14	0.55	0.53
3′,5′-Di-O-tetrahydropyranyl-ara-C (II)	0.35	0.48	0.78	0.92	—
2′-O-Methyl-ara-C (IVa)	0.05	0.11	0.32	0.70	0.61
2′-O,N^4-Dimethyl-ara-C (IVb)	0.11	0.29	0.49	0.73	0.66
2′-O,N^4,N^4-Trimethyl-ara-C (IVc)	0.27	0.37	0.63	0.77	0.70
Ara-U	0.10	0.27	0.25	0.43	0.54
2′-O-Methyl-ara-U (V)	0.32	0.42	0.46	0.51	0.66
2′-O,N_3-Dimethyl-ara-U (VII)	0.35	0.54	0.65	0.81	0.82

3′,5′-Di-O-tetrahydropyranyl-1-β-(D-arabinofuranosyl)cytosine (II). To 2.9 g (11 mmol) of the HCl salt of 2,2′-anhydro-ara-C (I), suspended in 60 ml of freshly distilled anhydrous dimethylformamide, was added 1.25 g p-toluenosulfonic acid monohydrate and 12.5 ml 2,3-dihydropyran (~140 mmol). The mixture was stirred with a magnetic flea at room temperature, with maintenance of strictly anhydrous conditions. Clarification of the suspension occurred during the first few hours. Stirring was continued for three days, following which the reaction was terminated by addition of 2 M-sodium methoxylate to pronounced alkalinity, to hydrolyse the 2,2′ bond. Removal of solvent under reduced pressure gave an oily brown residue, which was dissolved in water and extracted several times with ethyl ether to remove polymerization products of 2,3-dihydropyran. The aqueous phase was then extracted with 6×100 ml chloroform. The chloroform extracts were washed with water, dried over anhydrous $MgSO_4$ and brought to dryness under reduced pressure, yielding II in the form of a brownish oil (3.7 g, 82%). The oil crystallized slowly from ethyl acetate in the form of minute, colourless needles, m.p. 187 - 188.5°C. The crystalline product was chromatographically homogeneous on paper and on silica gel with all solvent systems employed.

Elementary analysis: for $C_{19}H_{29}N_3O_7$,
     calculated: C, 55.47; H, 7.06; N, 10.22,
     found: C, 55.50; H, 7.00; N, 10.05.

UV spectral data: $\lambda_{max}^{MeOH} = 273$ nm $(\varepsilon = 9.12 \times 10^3)$
          $\lambda_{min}^{MeOH} = 252$ nm $(\varepsilon = 6.29 \times 10^3)$
          $\lambda_{max}^{pH\ 7-12} = 271$ nm $(\varepsilon = 9.64 \times 10^3)$
          $\lambda_{min}^{pH\ 7-12} = 250$ nm $(\varepsilon = 6.13 \times 10^3)$

HCl salt of 2′-O-methyl-1-β-(D-arabinofuranosyl)cytosine (IVa·HCl). To 1.3 g (3 mmol) of II, dissolved in a mixture of 50 ml 1 N-KOH and 10 ml of 1,2-dimetho-

xyethane, and vigorously stirred, was added dimethylsulphate and 10 N-KOH, portionwise, to a total of 2 ml DMS and 4 ml of 10 N-KOH. The course of the reaction was followed after each addition by chromatography on HF_{254} silica gel with 85:15 chloroform-methanol. The reaction mixture was then extracted with 5×100 ml chloroform. The combined extracts were washed with water, dried with anhydrous $MgSO_4$, and brought to dryness to yield 1.2 g of a yellow oil (IIIa,b,c). This was dissolved in 20 ml of 50% acetic acid and 5 ml 1,2-dimethoxyethane and stored at 50°C for 2 days. Acetic acid was then removed by several evaporations from water, following which 5 ml 2 N-HCl was added and the solution brought to dryness. The residue was taken up in anhydrous ethanol, brought to dryness, and this step repeated several times. The final yellow residue was crystallized from anhydrous ethanol to give chromatographically homogeneous IVa·HCl in the form of small colourless needles (300 mg, 37%), m.p. 193 - 194.5°C (reported as 196 - 199°C by Darżynkiewicz et al., 1972).

UV spectral data: $\lambda_{max}^{pH\ 2} = 280$ nm $(\varepsilon = 12.35 \times 10^3)$
$\lambda_{min}^{pH\ 2} = 241$ nm $(\varepsilon = 1.51 \times 10^3)$
$\lambda_{max}^{pH\ 12} = 271$ nm $(\varepsilon = 8.64 \times 10^3)$
$\lambda_{min}^{pH\ 12} = 250$ nm $(\varepsilon = 5.71 \times 10^3)$

2'-O-*Methyl-ara-U* (*V*). A solution of 5 mg of the HCl salt of 2'-O-methyl-ara-C in 100 μl of 2.5 M-$NaHSO_3$ was left overnight at 37°C. The mixture was then made alkaline by addition of 2 N-NaOH. Chromatography on silica gel and on paper with various solvents showed only one spot, corresponding to 2'-O-methyl-ara-U, confirmed by the ultraviolet absorption spectrum of the eluate. This solution was now used as a control for identification of the products of deamination of product 1 (see p. 80).

2'-O-*Methyl-ara-U* (*V*) *and* 2'-*O,N*$_3$-*dimethyl-ara-U* (*VII*) *from product 1*. Product 1 (500 mg) was dissolved in 15 ml of 2.5 M-$NaHSO_3$ and the solution left overnight at 37°C. It was then brought to room temperature and an excess of 1 M-$Ba(OH)_2$ added to precipitate sulfite. After 30 min, the precipitate was filtered off and washed with water. The combined filtrates were passed through a 23×1.4 cm column of Dowex-50W(H$^+$) (200 × 400). The eluate was concentrated under vacuum to a yellow oil, which was subjected to preparative chromatography on PF_{254} gel with solvent *A1*. Two bands were obtained, each of which was eluted with methanol and crystallized from anhydrous ethanol.

One of these was 2'-O-methyl-ara-U, $R_F = 0.42$ with solvent *A2*, in the form of small colourless prisms, yield 47 mg (6%), m.p. 156 - 158°C.

UV spectral data: $\lambda_{max}^{pH\ 2\text{-}7} = 262$ nm $(\varepsilon = 10.64 \times 10^3)$
$\lambda_{min}^{pH\ 2\text{-}7} = 230$ nm $(\varepsilon = 2.17 \times 10^3)$
$\lambda_{max}^{pH\ 12} = 262$ nm $(\varepsilon = 7.97 \times 10^3)$
$\lambda_{min}^{pH\ 12} = 242$ nm $(\varepsilon = 5.38 \times 10^3)$

The other was 2'-*O,N*$_3$-dimethyl-ara-U, $R_F = 0.54$ with solvent *A2*, as colourless needles, yield 140 mg (19%), m.p. 198 - 199°C (reported as 198 - 200°C by Codington et al., 1968).

UV spectral data: $\lambda_{max}^{pH\ 2\text{-}12} = 261$ nm $(\varepsilon = 9.40 \times 10^3)$

$\lambda_{min}^{pH\ 2\text{-}12} = 232$ nm $(\varepsilon = 2.81 \times 10^3)$

Analysis of mother liquors from crystallization of IVa. The mother liquors remaining following crystallization of IVa, above, were brought to alkaline reaction with 2 N-NH_4OH and chromatographed on a preparative scale on PF_{254} silica gel with solvent *A1*. Four intense bands were located with a dark UV lamp. These were eluted with methanol and each was subsequently shown to be chromatographically homogeneous with both solvents *A1* and *A2*. They were classified as follows:

1. A mixture of 2'-*O*-methyl-ara-C (IVa) and 2'-*O*,N_3-dimethyl-ara-C (VI), $R_F = 0.11$, 500 mg. These two compounds could not be separated chromatographically, and the presence and identity of VI was established only after deamination (see above).

2. 2'-*O*,N^4-Dimethyl-ara-C (IVb), $R_F = 0.29$. This product was crystallized from anhydrous ethanol as the HCl salt, yield 40 mg, m.p. 165 - 167°C.

UV spectral data: $\lambda_{max}^{pH\ 2}\quad = 281$ nm $(\varepsilon = 14.40 \times 10^3)$

$\lambda_{min}^{pH\ 2}\quad = 242$ nm $(\varepsilon = 2.46 \times 10^3)$

$\lambda_{max}^{pH\ 7\text{-}12} = 270$ nm $(\varepsilon = 11.64 \times 10^3)$

$\lambda_{min}^{pH\ 7\text{-}12} = 250$ nm $(\varepsilon = 7.90 \times 10^3)$

3. 2'-*O*,N^4,N^4-Trimethyl-ara-C, $R_F = 0.37$, in the form of a pale yellow oil, yield 60 mg, which could not be crystallized, but was chromatographically homogeneous.

UV spectral data: $\lambda_{max}^{pH\ 2}\quad = 286\text{-}7$ nm

$\lambda_{min}^{pH\ 2}\quad = 247$ nm

$\lambda_{max}^{pH\ 7\text{-}12} = 277\text{-}8$ nm

$\lambda_{min}^{pH\ 7\text{-}12} = 243$ nm

4. 2'-*O*,N_3-Dimethyl-ara-U, $R_F = 0.54$, as colourless needles, yield 8.5 mg, m.p. 198 - 200°C (reported as 198 - 200°C by Codington *et al.*, 1968).

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REZPOŚREDNIA SYNTEZA 2'-O-METYLOWYCH ANALOGÓW 1-β-(D-ARABINOFU-BANOZYDU) CYTOZYNY I URACYLU ORAZ NIEKTÓRYCH ICH POCHODNYCH

Streszczenie

1. Opisano jednoznaczną syntezę 1-β-(2-O-metylo-D-arabinofuranozydu) cytozyny. 2'-O-Metylo-ara-C otrzymano działając siarczanem dwumetylu w alkalicznym roztworze wodnym na 3',5'-dwu-O-czterohydropiranylową pochodną ara-C, a następnie usuwano na drodze kwaśnej hydrolizy piranylowe grupy chroniące. 2'-O-Metylo-ara-U otrzymano następnie przez dezaminację pochodnej ara-C.

2. W stosowanych warunkach reakcji w znacznym stopniu (ok. 5%) zachodzi metylowanie grupy aminowej cytozyny, co zostało wykorzystane do otrzymania N^4-metylo i N^4,N^4-dwumetylo pochodnych 2'-O-metylo-ara-C. Ponadto, w przeciwieństwie do pochodnych metylowych cytydyny, u około 25% produktów metylowania ara-C w środowisku alkalicznym grupy metylowe były podstawione w pozycji N_3 cytozyny.

3. Wynikiem nietrwałości 1,3-dwupodstawionych pochodnych cytozyny w środowisku alkalicznym było pojawienie się wśród produktów reakcji 2'-O,N_3-dwumetylo-ara-U, który również wyodrębniono.

Z. KAZIMIERCZUK, ANNA PSODA and D. SHUGAR

ULTRAVIOLET AND INFRARED SPECTRAL STUDIES ON THE STRUCTURE AND TAUTOMERIC FORMS OF 2-THIOBARBITURIC ACIDS AND 2-THIOBARBITALS

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1. Ultraviolet absorption spectra in aqueous medium are reported for the neutral and ionic forms of 2-thiobarbituric acid and its mono- and di-*N*-methyl derivatives, as well as for some corresponding 5,5′-dialkyl pharmacologically important thiobarbitals. The pK values for the ionic equilibria of the various compounds were determined by spectral titration. 1,3-Dimethyl-5,5′-di-(*n*-propyl)-2-thiobarbituric acid exhibits an apparent ionic equilibrium with a pK about 10.7; this surprising result was shown to be due, not to ionization, but to reversible alkali-catalysed opening of the 1,6 bond of the pyrimidine ring. 2. Infrared absorption spectra in aqueous (D_2O) medium are reported for the anionic forms of 2-thiobarbituric acid and its *N*-methylated derivatives. Infrared (and ultraviolet) absorption spectra in chloroform solution were also determined for 1,3-dimethyl-2-thiobarbituric acid and several 2-thiobarbitals. Some of the infrared band assignments have been made for the neutral and anionic forms of several of the above compounds, and attention drawn to the asymmetry of the carbonyl stretching frequencies of the neutral forms. 3. The overall data were utilized to unequivocally establish the neutral, monoanionic and dianionic forms of thiobarbituric acids and thiobarbitals. 4. A relatively simple synthesis of the little known 1,3-dimethyl-2-thiobarbituric acid, a key compound in this investigation, is described.

The widespread interest in thiobarbiturates stems in large part from their pharmacological activity, due mainly to their enhanced lipid solubility as compared to the corresponding parent barbiturates (Mautner & Clayton, 1959), resulting from the replacement of the oxygen at position 2 by the less electronegative sulphur. The sodium salts of 2-thiobarbitals are widely employed as intravenous anaesthetics, although relatively little is as yet known regarding the molecular basis of their activity. Thiobarbiturates with an unsubstituted 5 position are also known to form polymethine dyes with lipids, as well as with 2-substituted pyrimidines, the analytical applications of which are quite extensive, and the mechanisms of formation of which have been investigated in some detail (see, e.g. Shepherd, 1964).

Notwithstanding their biological and clinical importance, it is surprising that the tautomeric forms of this class of compounds have not, to our knowledge, been adequately investigated or established. In particular, the preparation of one of the key derivatives necessary for this purpose, viz. 1,3-dimethyl-2-thiobarbituric acid, has been reported only once in connection with a study on the structure of pyrimidine polymethine dyes (Shepherd, 1964), although the corresponding 1,3-dimethyl-2-thiobarbitals have been known for some time (Brown, 1962) and 1,3-diphenyl-2-thiobarbituric acid was reported many years ago (Isherwood, 1909).

The present communication reports an improved and simple synthesis of 1,3-dimethyl-2-thiobarbituric acid and its use, along with other known 2-thiobarbiturates, for determination of the tautomeric forms of the parent 2-thiobarbituric acid by means of optical (UV and IR spectroscopy) methods. This, in turn, has made it possible to derive some concrete conclusions regarding the tautomeric forms of the pharmacologically active 5,5'-substituted 2-thiobarbituric acids, i.e. the 2-thiobarbitals, such as the well-known pentothal, 5-ethyl-5'-(1-methylbutyl)-2-thiobarbituric acid.

During the initial stages of this study, a paper appeared by Smyth *et al.* (1970) dealing with the electrochemical reduction properties of substituted 2-thiobarbiturates. On the basis of the pH-dependent changes of the UV absorption spectra and polarographic reduction waves for different analogues, it was postulated that 5,5'-dialkyl-2-thiobarbiturates may exist as four different ionic species in aqueous medium, from the diprotonated, through the monoprotonated and zwitterionic, to the monoanionic. These conclusions are at variance with previously reported findings for 5,5'-dialkylbarbiturates (Fox & Shugar, 1952) and have therefore been examined in some detail, below.

MATERIALS AND METHODS

2-Thiobarbituric acid, a B.D.H. (Poole, Dorset, Great Britain) product, was recrystallized several times from water and ethanol; 1(3)-methyl-2-thiobarbituric acid was synthesized according to the procedure of Cook *et al.* (1949).

1,3-*Dimethyl-2-thiobarbituric acid* was prepared by the condensation of *N,N'*-dimethylthiourea with malonic acid as follows: 5 g (0.048 M) of symm. dimethylthiourea and 7 g (0.07 M) malonic acid in 15 ml acetic acid was heated at 60 - 65°C with continuous stirring. To the heated reaction mixture was added, dropwise, 10 ml acetic anhydride. The temperature was then raised to 95°C and kept at this level for 3 h. The reaction mixture was evaporated to dryness under reduced pressure to give an oil which was heated to boiling in 25 ml methanol, and then left overnight at room temperature. The resulting precipitate was filtered off and recrystallized from methanol to give 3.3 g, m.p. 183 - 187°C. The combined filtrates from the previous operations were concentrated to provide an additional 1.2 g, m.p. 179 - 185°C (total yield 75%). An analytical sample, prepared by crystallization from 50% ethanol, was in the form of needles, m.p. 184 - 186°C. The product was chromatographically homogeneous. Elementary analysis gave:

Calculated: C, 41.86; H, 4.65; N, 16.27;
Experimental: C, 42.65; H, 4.60; N, 15.48.

Pentothal (*thiopental*), the sodium salt of 5-ethyl-5'-(1-methylbutyl)-2-thiobarbituric acid, a commercial sample, was recrystallized several times from ethanol. 1,3-Dimethyl-5,5'-di-(*n*-propyl)-2-thiobarbital was kindly provided by Dr. F. L. Rose of Imperial Chemical Industries Ltd. (Macclesfield, Cheshire, Great Britain) and 1,5,5'-tri-(*n*-propyl)-2-thiobarbituric acid, prepared by condensation of diethyl di-*n*-propyl cyanoacetate with *N*-*n*-propylthiourea in ethanolic sodium hydroxide, and acid hydrolysis of the resulting iminothiobarbituric acid, was a gift of Dr. M. E. Peel of Allen and Hanburys Ltd. (Ware, Hertfordshire, Great Britain).

Paper chromatography was ascending, with Whatman paper no. 1, and the following solvent systems:
  A) water-saturated *n*-butanol;
  B) *n*-butanol - water - acetic acid, 7:1:1 (by vol.);
  C) *n*-propanol - water - NH_4OH, 7:1:2 (by vol.).
Spots were revealed with the aid of a dark UV lamp.

Table 1

R_F *values for 2-thiobarbituric acids, ascending chromatography with Whatman paper no. 1 and three solvent systems*

Compound	R_F in solvent		
	A	B	C
2-Thiobarbituric acid	0.25	0.33	0.50
1(3)-Methyl-2-thiobarbituric acid	0.57	0.67	0.79
1,3-Dimethyl-2-thiobarbituric acid	0.66	0.88	0.84

All pH measurements were made with a Radiometer type PHM22 instrument. Buffers employed were usually 0.01 M, unless otherwise indicated. 1 N, 0.1 N and 0.01 N-HCl solutions were taken as pH 0, 1 and 2, respectively; and 0.01 N, 0.1 N and 1 N-NaOH as pH 12, 13 and 14. Although pH readings were obtained with an accuracy of 0.01 units, the spectrophotometrically titrated pK values are considered to be accurate to 0.05 pH units.

Ultraviolet absorption spectra were run on a Zeiss (Jena, G.D.R.) VSU-2 instrument, using 10-mm path-length cuvettes.

Infrared spectra were recorded on a Zeiss (Jena) model UR-10 spectrophotometer, using an NaCl prism for the region 1700 - 1300 cm^{-1} and an LiF prism for the 3600 - 2800 cm^{-1} region. Variable path-length cuvettes were fitted with CaF$_2$ windows. The D$_2$O employed originated from Koch-Light (Colnbrook, Great Britain) and was ⩾99.7% atom D. The chloroform used as solvent was a product of Riedel-De Haln AG (Seelze-Hanover, G.F.R.) and was treated with water to remove traces of ethanol, then dried over CaH$_2$.

RESULTS AND DISCUSSION

Ultraviolet absorption spectra of 2-tiobarbituric acids

1,3-*Dimethyl-2-thiobarbituric acid*. The absorption spectra of this compound as a function of pH are shown in Fig. 1. It is clear, from the isosbestic point at 246.5 nm, that only one dissociating function exists over the pH range 0 - 14; and, as in the case of 1,3-dimethylbarbituric acid (Fox & Shugar, 1952), this must involve a methylenic hydrogen at the 5-position. The calculated pK_1 value is 2.60 (Table 2) as compared to 4.6 for 1,3-dimethylbarbituric acid (Fox & Shugar, 1952). As in the case of 1,3-dimethylbarbituric acid, the compound is fully stable in strongly alkaline medium. The dissociation pattern for 1,3-dimethyl-2-thiobarbituric acid is therefore as in Scheme 1.
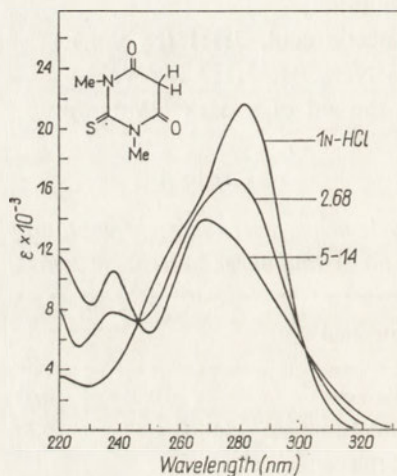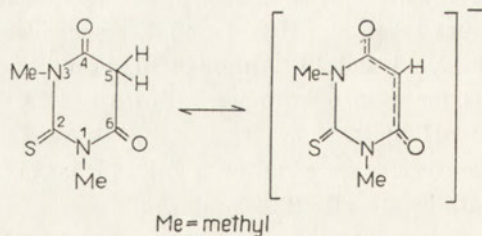

Fig. 1. UV spectra of 1,3-dimethyl-2-thiobarbituric acid at pH values indicated beside each curve. Isosbestic point at 246.5 nm indicates one ionic equilibrium with pK_1 2.60.

Me = methyl

Scheme 1

1(3)-*Methyl-2-thiobarbituric acid*. The absorption spectra of this derivative over the pH range 0 - 14 demonstrate the existence of two equilibria, which are shown in Figs. 2a and 2b. The first of these, with a pK_1 of 2.50 (Table 2), exhibits spectra for the neutral and monoanionic forms strikingly similar to those for 1,3-dimethyl-

Table 2

pK values (±0.05) for 2-thiobarbiturates and corresponding barbiturates and their N-alkyl derivatives, at 20°C

| Compound	pK_1	pK_2
Barbituric acid	3.9*	12.8*
2-Thiobarbituric acid	2.35[a]	11.3[b]
1(3)-Methylbarbituric acid	4.2*	12.8*
1(3)-Methyl-2-thiobarbituric acid	2.50	11.4
1,3-Dimethylbarbituric acid	4.60*	—
1,3-Dimethyl-2-thiobarbituric acid	2.60	—
5,5′-Diethylbarbituric acid (barbital)	7.85*	12.7*
5-Ethyl-5′-(1-methylbutyl)-2-thiobarbituric acid	7.57	12.7[c]
1(3)-Methylbarbital	8.45*	—
1,5,5′-Tri-(*n*-propyl)-2-thiobarbituric acid	8.15	—

* Values from Fox & Shugar (1952).
[a] 3.75 by potentiometric titration (Mautner & Clayton, 1959).
[b] 10.77 at 28°C by spectral titration (Sato, 1957).
[c] 12.07 at 28°C by spectral titration (Sato, 1957).

2-thiobarbituric acid (Fig. 1), allowing for the bathochromic effects due to methylation. This equilibrium must therefore correspond to dissociation of one of the methylenic hydrogens. The second equilibrium, with a pK_2 value of 11.4, should consequently be due to dissociation of the $N_{3(1)}$ hydrogen. The resultant decrease in extinction coefficient of the long-wavelength absorption band is what would be anticipated from the increase in aromaticity of the heterocyclic ring, the overall dissociation pattern being as in Scheme 2.

Me = methyl
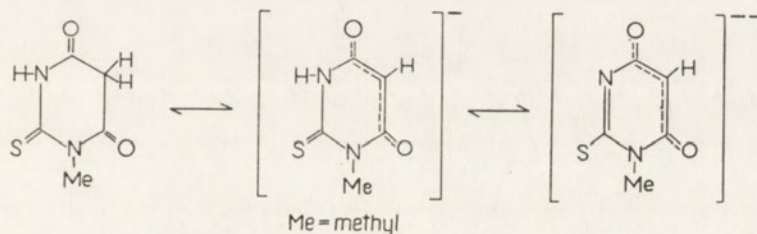
Scheme 2

2-Thiobarbituric acid. This compound likewise exhibits two equilibria over the accessible pH range, each of which is shown separately in Figs. 3a and 3b. The first of these, with a pK_1 of 2.35 (Table 2), obviously corresponds to the first equilibrium for 1(3)-methyl-2-thiobarbituric acid (Fig. 2a) as well as to that for 1,3-dimethyl-2-thiobarbituric acid (Fig. 1) and involves formation of the monoanion from the neutral form. The second, Fig. 3b, is fully analogous to the second equilibrium exhibited by 1(3)-methyl-2-thiobarbituric acid, Fig. 2b, absent in 1,3-dimethyl-2-thiobarbituric acid (Fig. 1). The second equilibrium therefore corresponds to formation of the dianion from the monoanion (Scheme 3).
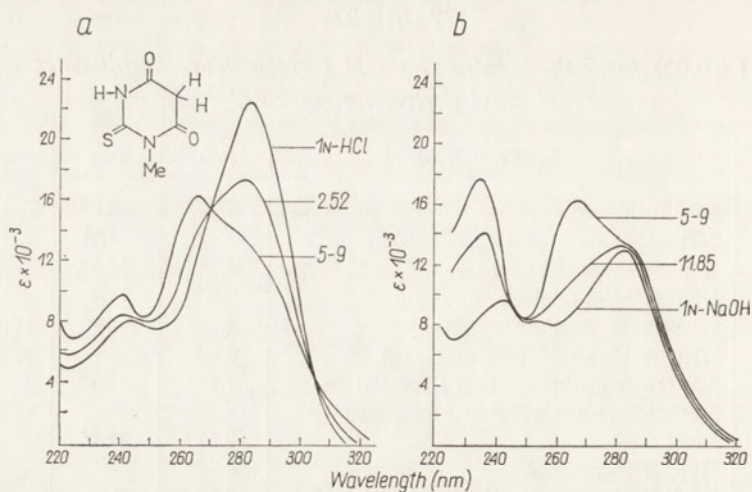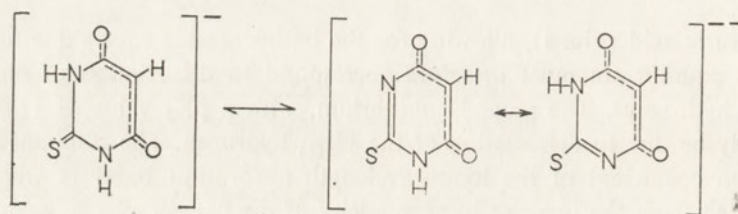
Fig. 2. UV spectra of 1(3)-methyl-2-thiobarbituric acid at pH values indicated, demonstrating two ionic equilibria: *a*, transition from neutral to monoanionic form, $pK_1 = 2.50$; *b*, transition from monoanion to dianion, $pK_2 = 11.4$.
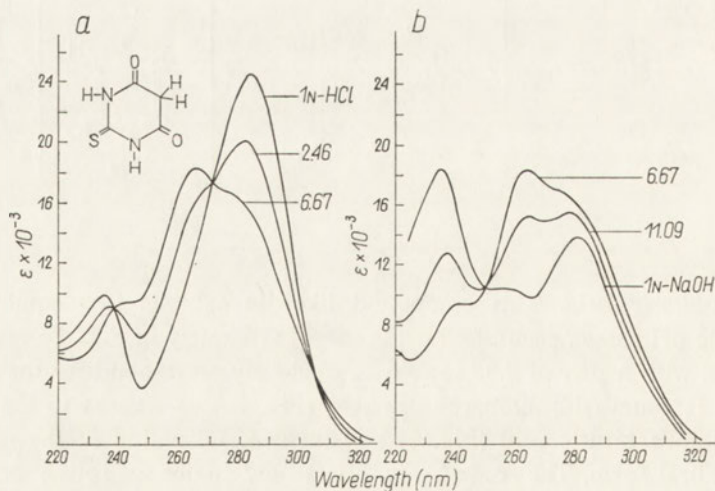

Scheme 3

Fig. 3. UV spectra of 2-thiobarbituric acid at pH values indicated, demonstrating two ionic equilibria: *a*, transition from neutral form to monoanion, $pK_1 = 2.35$; *b*, transition from monoanion to dianion, $pK_2 = 11.3$.

The pronounced increase in acidities associated with monoanion formation in 2-thiobarbituric acid and its *N*-methyl derivatives, relative to those for the corresponding barbituric acids (Table 2), is undoubtedly related to the higher polarizability of the carbon-sulphur double bond as compared to the carbon-oxygen double bond. It should be noted that Mautner & Clayton (1959) reported a pK_1 of 3.75 for 2-thiobarbituric acid, by potentiometric titration, a value not much different from that for barbituric acid (see Table 2). This erroneous finding, together with similar errors in pK measurements for other thio- and selenobarbiturates, led the foregoing authors to draw ambiguous conclusions even for the neutral forms of thiobarbiturates, which they postulated as existing in the 2,4-diketo-6-hydroxy form. This proposal is rendered invalid not only by the results cited above but, as will be shown below, by an examination of the infrared spectra.

Ultraviolet absorption spectra of 5,5'-dialkyl-2-thiobarbiturates

The pH-dependence of the UV absorption spectra, and the associated ionic equilibria, for 5-ethyl-5'-(1-methylbutyl)-2-thiobarbituric acid, the mono-*N*-alkyl analogue 1,5,5'-tri-(*n*-propyl)-2-thiobarbituric acid, and the di-*N*-alkyl derivative 1,3-dimethyl-5,5'-di-(*n*-propyl)-2-thiobarbituric acid are exhibited in Figs. 4, 5, and 6, respectively.

At pH 1 - 5 all three compounds possess a long wavelength absorption band at about 285 nm, the extinction coefficient of which increases with *N*-alkylation; and a short wavelength band, the location of which is shifted to the red on *N*-alkylation, passing from 237 nm for the non-alkylated derivative (Fig. 4a) to 250 nm for the mono-*N*-alkylated (Fig. 5) and, finally, to about 260 nm for the di-*N*-alkyl
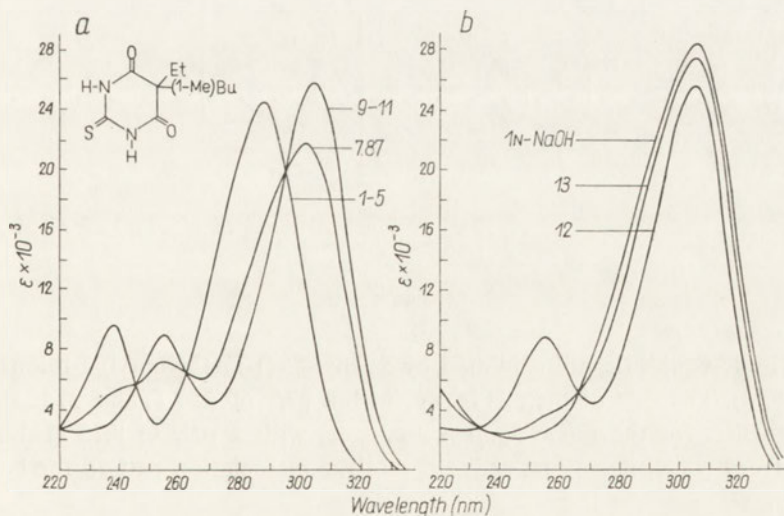

Fig. 4. UV spectra of pentothal, 5-ethyl-5'-(1-methylbutyl)-2-thiobarbiturate, at pH values indicated, and showing two ionic equilibria: *a*, transition from neutral to monoanionic form, $pK_1 = 7.57$; *b*, transition from monoanionic to dianionic form, $pK_2 = 12.7$.

compound, where it overlaps with the long wavelength band (Fig. 6). This behaviour is fully consistent with a common skeletal structure for the neutral forms of all three derivatives, as illustrated in the figures.
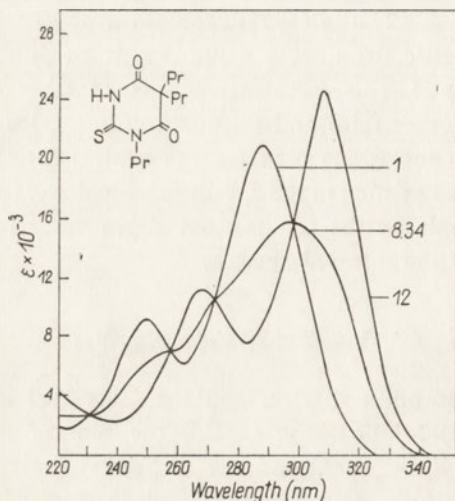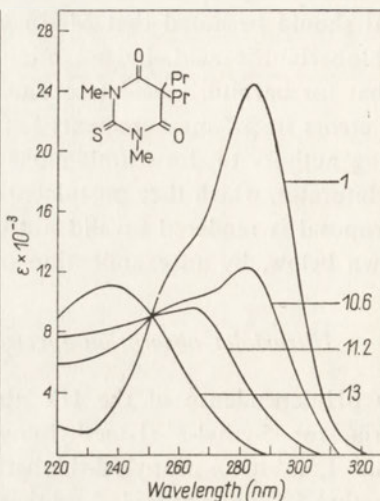

Fig. 5   Fig. 6

Fig. 5. UV spectra, at pH values indicated, of 1,5,5'-tri-(n-propyl)-2-thiobarbituric acid, demonstrating the existence of one ionic equilibrium with pK_1 8.15.

Fig. 6. UV spectra, at pH values indicated, of 1,3-dimethyl-5,5'-di-(n-propyl)-2-thiobarbituric acid. Note that this spectral pattern is not due to an ionic equilibrium, but to reversible ring opening as a function of pH (see Text for details).

R_1 = H or alkyl
R_2, R_3 = alkyl
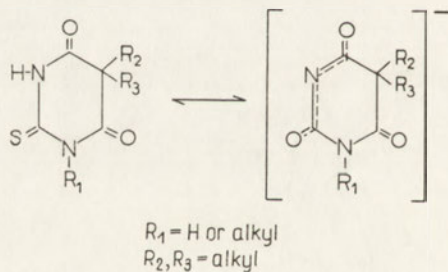
Scheme 4

Two ionic equilibria are exhibited by 5-ethyl-5'-(1-methylbutyl)-2-thiobarbituric acid (Fig. 4). The first of these, Fig. 4a, with a pK_1 of 7.57 (Table 2) is strikingly similar to that for the mono-N-alkyl analogue, with a pK_1 of 8.15 (Table 2 and Fig. 5), from which it may be deduced that the dissociation pattern for both is as shown in Scheme 4.

The single apparent ionic equilibrium for the di-N-alkyl derivative (Fig. 6), which possesses no dissociable hydrogens, requires special consideration and is discussed in detail in a separate section, below.

Direct evidence for the charge delocalization between the S and O atoms in Scheme 3 is lacking. It is analogous to that postulated for a 2-thiouracil derivative (Shugar & Fox, 1952a) and to that established by infrared spectroscopy for 2,4-diketopyrimidines (Wierzchowski *et al.*, 1965). Direct evidence for charge delocalization in the monoanion shown in Scheme 4 would probably be forthcoming by means of Raman spectroscopy. Unfortunately the solubility of this compound is so low as to render such measurements out of the question.

As concerns the second ionic equilibrium for pentothal (Fig. 4), no suitable reference compound is available to unequivocally establish the structure of the dianion. It appears logical, however, to assume that it must involve more extensive charge delocalization, as shown in Scheme 5.


R_1 = ethyl
R_2 = 1-methylbutyl
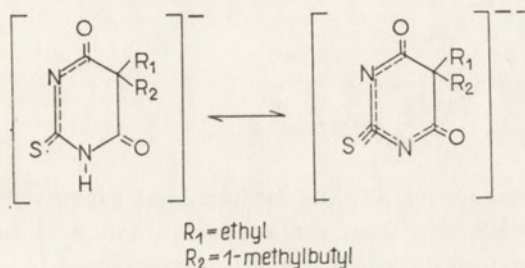
Scheme 5

1,3-Dimethyl-5,5'-di-(n-*propyl*)*-2-thiobarbituric acid.* The absorption spectrum of this compound is invariant over the pH range 0 to 8.5 (Fig. 6), as might have been anticipated, since it contains no functional group capable of undergoing dissociation. Consequently, and notwithstanding that this had been previously reported by Smyth *et al.* (1970), it was surprising to note that increasing the pH above 8.5 led to apparent "titration" of some functional group, with a "pK" of 10.7, in agreement with the value noted by Smyth *et al.* (1970). This striking observation led the foregoing authors to conclude that what we referred to above as the neutral form of the molecule is, in reality, the protonated form, and that the pK of 10.7 corresponds to deprotonation. If this were true, it would render completely invalid our own conclusions presented above for the other compounds in this series. We have therefore examined this problem in some detail, and now present evidence to the effect that the pK of 10.7 for 1,3-dimethyl-5,5'-di-(*n*-propyl)-2-thiobarbituric acid is *not* due to ionization of some functional group.

When a neutral solution of the foregoing compound was *rapidly* brought to a pH above 10, it was found that the absorption spectrum at a given pH value varied with time, as a function of temperature, finally reaching an equilibrium value which, at room temperature, required 2 - 3 minutes. When such a solution was *quickly* brought back within the pH range 3 - 7, several minutes were again required to attain the original spectrum of the neutral form.

The foregoing indicates that the pK of 10.7 is due, not to an ionic equilibrium, but to some pH-dependent chemical reaction. The reversibility of this reaction

pointed to ring opening as the most likely possibility. Reference should be made at this point to the known alkaline lability of dialkylated 2,4-diketopyrimidines (Shugar & Fox, 1952b) and, particularly, of 1,3-dimethylbarbital (Fox & Shugar, 1952). By analogy with the ring opening in alkaline medium of hydrated uridine, which is followed by dehydration and ring closure (Fikus & Shugar, 1966), reversible fission of the 1,6 (or equivalent 3,4)-bond seemed the most likely possibility (Scheme 6). Solubility considerations rendered it impossible to examine the infrared spectrum


Me = methyl
Pr = propyl
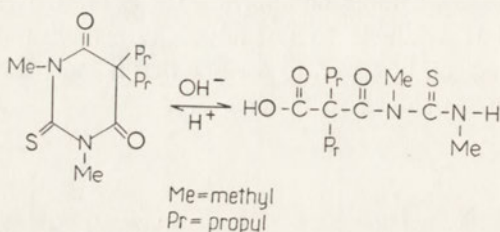
Scheme 6

of this compound in aqueous alkaline medium. But supporting evidence for this interpretation is forthcoming from studies on the nature of hydrolysis products of barbitals, the literature on which is extensively reviewed in a recent paper by Garrett et al. (1971). In particular, it has been shown that, in the presence of OH^- ions, reversible equilibria exist between some barbitals and their corresponding malonuric acids, via 1,6 ring opening (Garrett et al. 1971; Aspelund, 1969) as in Scheme 7. Furthermore, the fraction of the open-chain form is dependent on the pH.

R_1, R_2 = alkyl
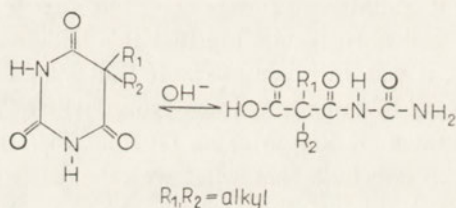
Scheme 7

In support of this, we have been informed by Dr M. E. Peel (personal communication) that alkaline hydrolysis of 5-ethyl-5'-isobutyl-1-methyl-2-thiobarbituric acid in carefully buffered solution leads to quantitative conversion to the open ring derivative (Scheme 8), which subsequently undergoes further slow hydrolysis under these conditions. These results, in conjunction with our observations on 1,3-dimethyl-5,5'-di-(n-propyl)-2-thiobarbituric acid, suggest that hydrolysis of 2-thiobarbitals involves mechanisms similar to those for barbitals, and warrant further study in view of the pharmacological importance of the thiobarbitals.

Comparison of neutral and anionic forms. Figures 7a, b and c exhibit, for comparison purposes, the UV spectra of the neutral, monoanionic and dianionic forms, respectively, of 2-thiobarbituric acid and its mono- and dimethyl derivatives, and pentothal.

Me = methyl
R₁ = ethyl
R₂ = isobutyl
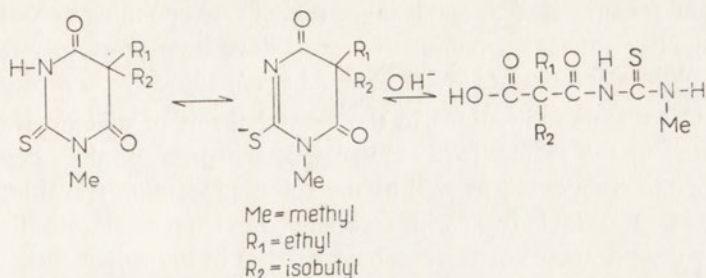
Scheme 8

Figure 7a clearly shows the similarity of the neutral forms for all four derivatives, while Fig. 7b and 7c demonstrate the similarities of the anionic forms for the three thiobarbiturates, and their dissimilarity from those for pentothal, in accordance
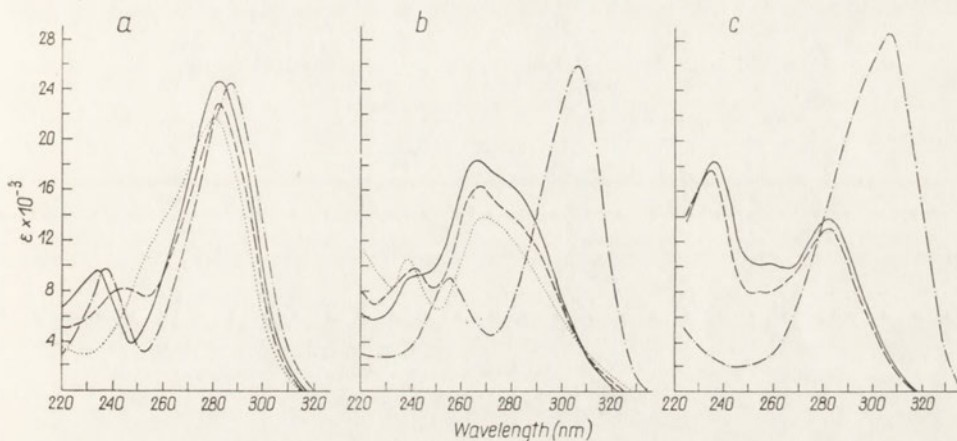
Fig. 7. UV absorption spectra of: *a*, neutral, *b*, monoanionic, and *c*, dianionic forms of: ——, 2-thiobarbituric acid; - - -, 1(3)-methyl-2-thiobarbituric acid; ···, 1,3-dimethyl-2-thiobarbituric acid; ·—·—·, pentothal (5-ethyl-5′-(1-methylbutyl)-2-thiobarbituric acid).

with the difference in structures of the monoanions of 2-thiobarbiturates (Schemes 1 and 2) relative to the 2-thiobarbital monoanions (Schemes 4 and 5), as well as the difference in structure of the 2-thiobarbiturate dianions (Schemes 2 and 3) relative to that for 2-thiobarbital (Scheme 5).

Infrared absorption spectra

These were seriously limited by solubility considerations to only several of the foregoing compounds, either in chloroform, or in D_2O at pD 8 and pD 13, i.e. the monoanionic and dianionic forms, the solubility of the neutral forms being too low in aqueous acid medium. Spectral modifications were recorded in the region corresponding to double bond stretching vibrations, and deformation and ring

vibrations; and for several compounds in the region corresponding to X-H stretching vibrations. Similar studies on tautomeric forms have been previously reported for various pyrimidines and purines (Wierzchowski *et al.*, 1965; Psoda & Shugar, 1971).

The $C=O$ stretching vibrations in the neutral forms of diketopyrimidines are usually located in the region $1670 - 1750 \text{ cm}^{-1}$, the precise values depending on the nature of the compound, as well as the solvent medium. Transition from the neutral to the monoanionic form with accompanying charge delocalization is manifested by the disappearance of the carbonyl band(s) in the neighbourhood of 1700 cm^{-1} and the appearance of new bands at frequencies below 1600 cm^{-1} (Psoda & Shugar, 1971).

Spectra in non-aqueous media. The spectrum of pentothal in chloroform is exhibited in Fig. 8a. That this represents, in fact, the neutral form is shown by the band at 3377 cm^{-1}, typical for the N-H stretch. In accordance with this, the two intense carbonyl frequencies expected for the neutral form are seen at 1707 and 1740 cm^{-1}.
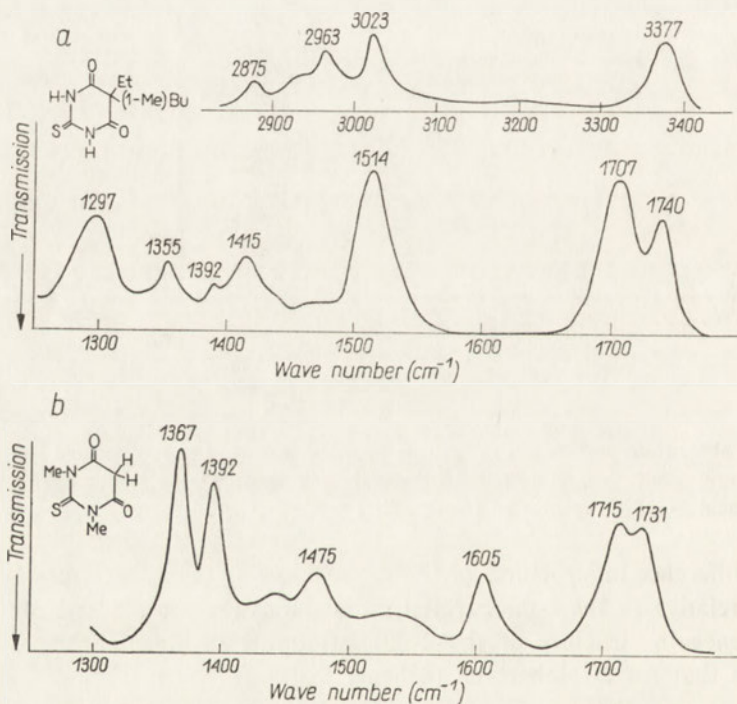
Fig. 8. IR spectra in chloroform of: *a*, pentothal (5×10^{-2} M); path length 50 μ; *b*, 1,3-dimethyl-2-thiobarbituric acid (4.0×10^{-3} M); path length 1 mm.

It proved possible also to examine the UV absorption spectrum of pentothal in the same chloroform solvent over the range $250 - 320 \text{ nm}$. This was almost identical with that for pentothal in aqueous medium over the pH range 1 - 7 (Fig. 4a), which was postulated above as corresponding to the neutral form. Consequently

the infrared and ultraviolet spectral data are in full accord, as regards the neutral forms of these compounds.

1,3-Dimethyl-2-thiobarbituric acid in chloroform (Fig. 8b) shows two well-resolved and intense carbonyl bands at 1715 and 1731 cm^{-1}. For 1,5,5'-tri-(n-propyl)-2-thiobarbituric acid these bands are located at 1699 and 1728 cm^{-1} (Fig. 9a), whereas in 1,3-dimethyl-5,5'-di-(n-propyl)-2-thiobarbituric acid they appear at 1690 and 1720 cm^{-1} (Fig. 9b). The observed shifts to lower frequencies on N-methylation are in agreement with the results of studies on carbonyl frequencies in a variety of pyrimidines, in all of which N-alkylation led to reductions in carbonyl frequencies (Wierzchowski, Litońska & Shugar, unpublished results). The more pronounced shift in these frequencies for 1,3-dimethyl-5,5'-di-(n-propyl)-2-thiobarbituric
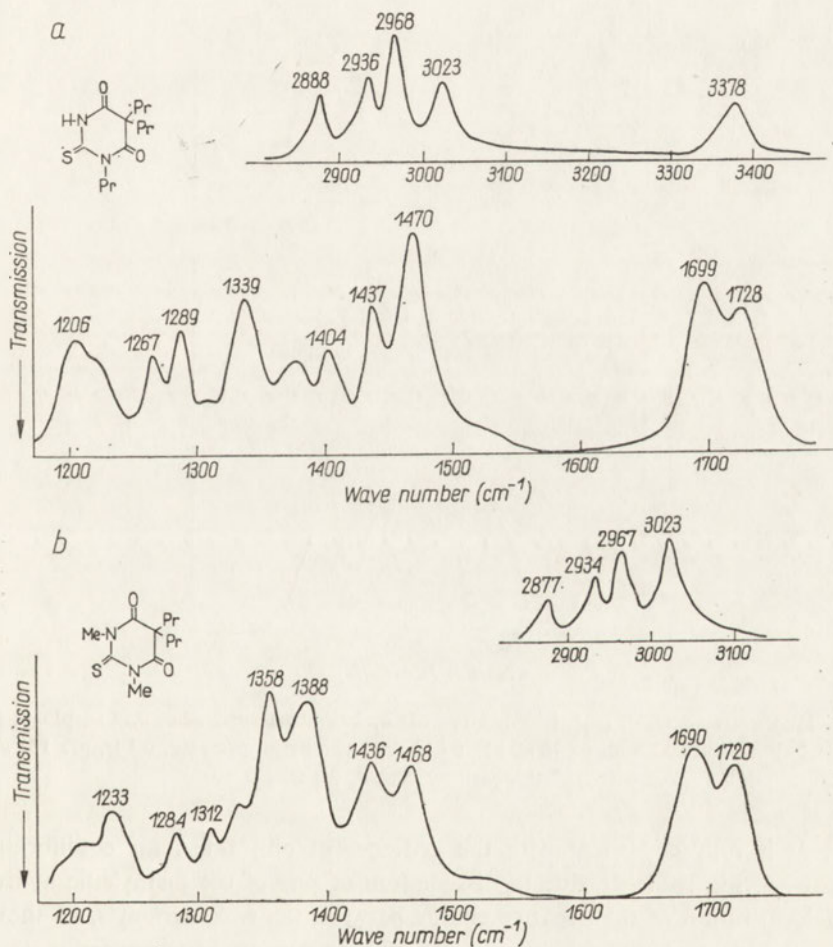

Fig. 9. IR spectra in chloroform of: a, 1,5,5'-tri-(n-propyl)-2-thiobarbituric acid (8.0×10^{-2} M), path length 50 μ; b, 1,3-dimethyl-5,5'-di-(n-propyl)-2-thiobarbituric acid (8.0×10^{-2} M), path length 50 μ.

acid suggests, furthermore, that this must be due in part to alkylation at the 5-position, since for pentothal the carbonyl frequencies are located at 1707 and 1740 cm^{-1}.

Spectra of monoanionic forms in aqueous medium. The spectrum of 1,3-dimethyl-2-thiobarbituric acid at about pD 8, i.e. of the monoanionic form, is presented in Fig. 10b. It shows two intense bands at 1379 and 1608 cm^{-1}, and the absence of any bands at higher frequencies which might conceivably be ascribed to carbonyl groups. Increasing the pD to 13 leaves the spectrum essentially unaltered. This
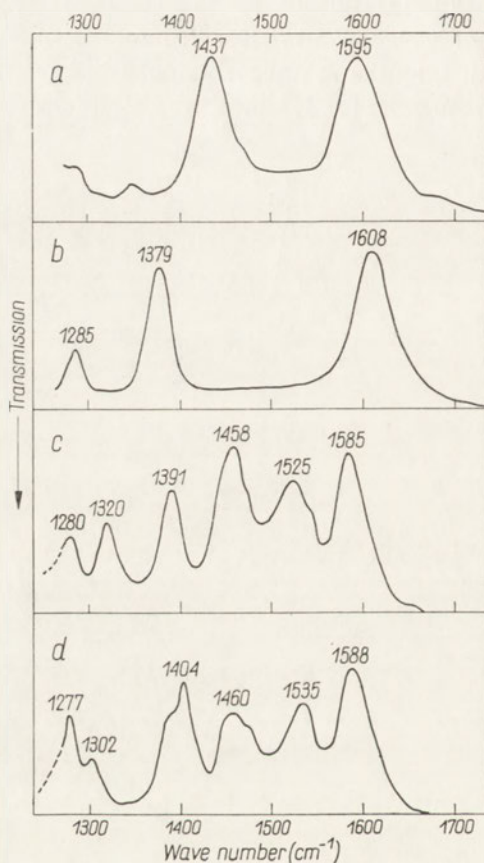
Fig. 10. IR spectra in D$_2$O, path length 50 μ, of: *a*, 2-thiobarbituric acid, 0.11 M, pD 8; *b*, 1,3-dimethyl-2-thiobarbituric acid, 0.076 M, pD 8 - 13; *c*, 2-thiobarbituric acid, 0.1 M, pD 13; *d*, 1(3)-methyl-2-thiobarbituric acid, 0.1 M, pD 13.

behaviour is fully consistent with the existence of only one ionic equilibrium (at pD below 8, see Table 2), due to dissociation of one of the methylenic hydrogens with delocalization of the negative charge between the two carbonyls, as shown in Scheme 1, and resulting in disappearance of both carbonyl frequencies.

The two intense bands at 1379 cm^{-1} ($\varepsilon^A \sim 2100$) and 1608 cm^{-1} ($\varepsilon^A \sim 3000$) correspond predominantly to the symmetric and asymmetric vibration frequencies of the groups (C══O)$^-$ in the system (O══C══C══C══O) with C$_{2u}$

symmetry. This interpretation is fully analogous to the situation for the mono-anions of 2,4-diketopyrimidines, such as uracil and thymine, in which the 5,6 bond is saturated (Wierzchowski *et al.*, 1965); e.g. in the case of uracil with a saturated 5,6 bond, the frequencies 1536 cm^{-1} ($\varepsilon^A \sim 1150$) and 1436 cm^{-1} ($\varepsilon^A \sim 850$) were assigned to the asymmetric and symmetric vibrations of C$=$O in the resonance bond system (O$=$C$=$N$=$C$=$O)$^-$, in accord with results previously obtained with analogous isolated systems such as the monoanions of acetylacetone and cyanoacetylacetone (Wierzchowski & Shugar, 1965).

The monoanion of 2-thiobarbituric acid itself at pD about 8 (Fig. 10a) exhibits asymmetric and symmetric frequencies at 1595 cm^{-1} ($\varepsilon^A \sim 2500$) and 1437 cm^{-1} ($\varepsilon^A \sim 2700$), both of which are somewhat displaced relative to the corresponding bands for 1,3-dimethylbarbituric acid monoanion as a result of *N*-alkylation. The spectra are entirely consistent with formally identical structures for the mono-anions of both these compounds, as shown in Schemes 1 and 3, and in agree-ment with the conclusions based on the UV spectra (see above).

Dianionic form of 2-thiobarbituric acid. The structure of the dianion of 2-thio-barbituric acid is readily forthcoming from a comparison of the spectrum of this compound at pD$\sim$13 (Fig. 10c) with that for the dianion of the corresponding 1(3)-methyl-2-thiobarbituric acid (Fig. 9d)[1]. The close similarity between the two is indeed striking, as is also their difference from that for the 2-thiobarbiturate monoanion (Fig. 10a), indicating that the additional negative charge resulting from dianion formation must be localized elsewhere than on one of the oxygens.

For 1(3)-methyl-2-thiobarbituric acid, the only possible form of the dianion is that illustrated in Scheme 2. The corresponding dissociation pattern for dianion formation in 2-thiobarbituric acid itself must consequently conform to that shown in Scheme 3, in agreement with the UV data.

Band assignments for thiobarbiturate anions

While the IR spectra of the thiobarbiturate anions permit of a reasonable estab-lishment of their structures, in agreement with those inferred from the UV spectra, unequivocal assignments for the various bands in these spectra is considerably more complex. In the case of the monoanions (Figs. 10a and 10b), the resonating system (O$=$C$=$C$=$C$=$O)$^-$ should reveal, in addition to the asymmetric and symmetric vibration frequencies of the (C$=$O)$^-$ groups, bands with lower intensity corresponding to the asymmetric and symmetric frequencies of the (C$=$C) groups (Wierzchowski *et al.*, 1965; Wierzchowski & Shugar, 1965). The apparent absence of these latter may be due to masking by the very intense bands corre-sponding to the (C$=$O)$^-$ groups.

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[1] The spectrum for the monoanion of this derivative could not be recorded because of its low solubility. However, the similarity of its UV spectrum (Fig. 2b) with that for the monoanion of 2-thiobarbituric acid (Fig. 3b), as well as the similarity of the pK_2 values for both (Table 2), leave no doubts as to the formal structural identity of the two monoanions.

In the case of the dianions the situation is rendered even more complex due to conjugation of the system $(O\!=\!=\!C\!=\!=\!C\!=\!=\!C\!=\!=\!O)^-$ with the $^-S\!-\!C\!=\!N$ bond system. This leads to shifts in the frequencies of the $(C\!=\!=\!O)^-$ groups relative to those for the monoanions (1437 and 1595 cm^{-1} for 2-thiobarbituric acid monoanion, and 1458 and 1585 cm^{-1} for 2-thiobarbituric acid dianion).

The band at 1525 cm^{-1} for 2-thiobarbituric acid dianion, shifted to 1535 cm^{-1} in 1-methyl-2-thiobarbiturate dianion, is most likely due to the $C\!=\!N$ stretching vibration.

It is clear that additional experimental data are essential for band assignments in these systems, and attempts are now under way to obtain such data by means of laser Raman spectroscopy.

Asymmetry of carbonyl stretching frequencies. The spectrum in chloroform solution of 1,3-dimethyl-5,5'-di-(*n*-propyl)-2-thiobarbituric acid is exhibited in Fig. 8b. It is unfortunate that this compound was insufficiently soluble in alkali to independently confirm the postulated ring opening under these conditions deduced from the UV absorption spectra, above. It is, however, of interest that the neutral form in chloroform, which possesses C_{2u} symmetry about the axis C_2—C_5, does not show two symmetrical carbonyl frequencies. This problem is not basic to the principal objective of this investigation, but is of some interest in relation to the interpretation of the carbonyl stretching frequencies.

1,3-Dimethyl-2-thiobarbituric acid (neutral form) with C_{2u} symmetry about the C_2—C_5 axis, possesses two $C\!=\!O$ frequencies of equal intensities, at 1715 and 1731 cm^{-1}, but only weakly split ($\Delta v\sim16\ cm^{-1}$), frequently observed in systems containing two symmetrically disposed carbonyls, due to resonance splitting (Herzberg, 1950; Kendall, 1966). In pentothal, Δv for the two carbonyls is somewhat larger, 30 cm^{-1}, and the short wavelength band is the more intense, so that even a slight perturbation of molecular symmetry leads to a pronounced effect on the spectrum, in agreement with the postulate that we are dealing with resonance splitting.

The lack of symmetry of the two carbonyl bands in 1,3-dimethyl-5,5'-di-(*n*-propyl)-2-thiobarbituric acid must, consequently, be due to reasons other than
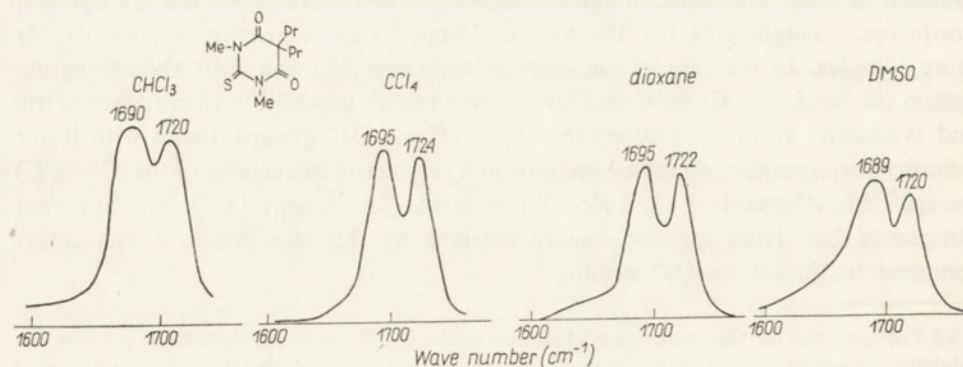

Fig. 11. IR spectra of 1,3-dimethyl-5,5'-di-(*n*-propyl)-2-thiobarbituric acid in various solvents in C=O stretching region.

in the case of pentothal. Although the splitting of the two bands ($\Delta v \sim 30$ cm^{-1}) is similar to that observed for the non-symmetrical pentothal (33 cm^{-1}), the long-wavelength band (1690 cm^{-1}) is more intense and broader than the short-wavelength one at 1720 cm^{-1}. Such asymmetry of carbonyl frequencies has been observed in a wide range of symmetrical derivatives of barbitals (Sucharda-Sobczyk, 1970; Bouche *et al.*, 1966) and its source has been ascribed to Fermi resonance, one of its characteristic features being its sensitivity to solvent effects. We have therefore recorded the spectrum of the foregoing analogue in a series of solvents (Fig. 11), from which it is seen that a decrease in dielectric constant of the solvent medium leads to an increase in symmetry of the two carbonyl frequencies.

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BADANIA SPEKTRALNE W ULTRAFIOLECIE I PODCZERWIENI STRUKTURY
I FORM TAUTOMERYCZNYCH KWASÓW 2-TIOBARBITUROWYCH I 2-TIOBARBITALI

Streszczenie

1. Podano widma absorpcyjne UV w roztworach wodnych form obojętnych i jonizowanych kwasu 2-tiobarbiturowego, jego mono- i dwu-N-metylopochodnych oraz jego niektórych pochodnych 5,5'-dwualkilowanych, ważnych z punktu widzenia farmakologicznego. Wartości pK tych związków wyznaczono metodą miareczkowania spektralnego. W wyniku miareczkowania spektralnego kwasu 1,3-dwumetylo-5,5'-dwu-n-propylo-2-tiobarbiturowego uzyskano wartość 10.7, która nie odpowiada równowadze jonowej lecz odwracalnemu alkalicznemu rozpadowi pierścienia pirymidynowego w pozycji 1,6.

2. Uzyskano widma IR anionów kwasu 2-tiobarbiturowego i jego N-metylowanych pochodnych w roztworach wodnych (D_2O) oraz widma IR i UV kwasu 1,3-dwumetylo-2-tiobarbiturowego i kilku 2-tiobarbitali w chloroformie. Częściowo przyporządkowano pasma obserwowane w widmach IR form obojętnych i zjonizowanych kilku badanych związków i zwrócono uwagę na asymetrię pasm odpowiadających drganiom rozciągającym grup karbonylowych.

3. Powyższe dane pozwoliły na jednoznaczne ustalenie form obojętnych, monoanionów i dwuanionów kwasów tiobarbiturowych i tiobarbitali.

4. Opisano prostą syntezę mało znanego kwasu 1,3-dwumetylo-2-tiobarbiturowego.

LUDWIKA ZAGÓRSKA and P. SZAFRAŃSKI

RIBOSOMAL BINDING SITE OF fMet-tRNA IN *ESCHERICHIA COLI* SYSTEM DIRECTED BY PHAGE f2 RNA

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1. Tetracycline at a concentration of 0.05 - 0.1 mм inhibits the phage f2 RNA-directed binding of Ala-tRNA to ribosomes but has no effect on the binding of fMet-tRNA. 2. Edeine inhibits the phage f2 RNA-dependent formation of the initiation complex. 3. When GMP-PCP was used instead of GTP, the binding of fMet-tRNA to ribosomes was not inhibited by tetracycline. However, the initiator tRNA attached under these conditions cannot serve as donor of the formylmethionyl group. 4. The obtained results suggest that fMet-tRNA, before correct positioning at P site, adopts within this site a transient conformation in which it is unable to react with puromycin and to initiate synthesis of the peptide chain.

According to Watson's hypothesis (Watson, 1964) the ribosome contains two functional sites for the binding of AA-tRNA[1]: the peptidyl site (P site) and amino-acyl site (A site). The A site is the one through which all the AA-tRNAs, required for elongation of the peptide chain, enter the ribosome-mRNA complex while the P site binds the growing peptidyl-tRNA. These sites may be distinguished by means of antibiotics such as puromycin, streptomycin, edeine or tetracycline. Puromycin is known to react with peptidyl-tRNA or initiator tRNA (fMet-tRNA) bound to the P site (Traut & Monro, 1964), streptomycin releases initiator tRNA from the P site (Lelong *et al.*, 1971) while edeine blocks the binding of peptidyl-tRNA (or initiator tRNA) to the P site (Szer & Kuryło-Borowska, 1970). Tetracycline specifically inhibits the attachment of AA-tRNA to the A site (Gottesman, 1967).

The mechanism of entry of initiator tRNA to the ribosome is not clear. This particular AA-tRNA must be bound to the P site where it serves as an aminoacyl donor in initiating protein chain elongation.

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[1] Abbreviations: fMet-tRNA, *N*-formylmethionyl transfer RNA; Ala-tRNA, alanyl transfer RNA; AA-tRNA, aminoacyl transfer RNA; GMP-PCP, guanylyl-5′-methylene diphosphonate; IF, initiation factors; EF T, elongation factor T.

The binding of fMet-tRNA to a ribosome-mRNA complex requires besides initiation factors also GTP or its non-splittable analogue, GMP-PCP (Anderson *et al.*, 1967). fMet-tRNA in initiation complex with GMP-PCP does not react with puromycin. This was interpreted as an indication of the binding of initiator tRNA to the A site prior to its binding to the P site (Sarkar & Thach, 1968). Previous studies (Zagórska *et al.*, 1971) on the formation of initiation complex in the presence of a synthetic template (poly AUG) showed that initiator tRNA is attached to the P site without preliminary interaction with the A site. In contrast to the synthetic template, initiation of translation of natural mRNAs could be affected by factor IF 3, which is involved in the reaction between mRNA and the ribosome and which might recognize nucleotide sequences proximal to the initiation codon (Revel *et al.*, 1970). The secondary structure of natural templates also seems to play an important role in the recognition of the initiation codon (Steitz, 1969).

In this work we have studied the formation of the initiation complex and the attachment site of fMet-tRNA on the ribosome in a system directed by phage f2 RNA. Puromycin, edeine and tetracycline were used to differentiate between the P and A sites. The results obtained confirmed the conclusions reached with synthetic templates and showed that in a system directed by f2 RNA the binding of fMet-tRNA to the P site does not proceed through the A site. It is proposed that the binding of initiator tRNA to the P site takes place through an intermediary conformational form of the fMet-tRNA-ribosome-f2 RNA complex.

MATERIALS AND METHODS

Chemicals. Casamino acids and yeast extract were purchased from Difco (Detroit, Mich., U.S.A.), 2-mercaptoethanol from Koch-Light Ltd. (Colnbrook, Bucks., England) and tris from Fluka A. G. (Buchs, Switzerland). *E. coli* tRNA (stripped) was obtained from General Biochemicals (Chagrin Falls, Ohio, U.S.A.), GTP, ATP, 5'-AMP, phosphoenolpyruvate and alumina type 305 from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). GMP-PCP was from Miles Laboratory (Elkhart, Ind., U.S.A.), dithiothreitol (DTT) from Calbiochem (Los Angeles, Calif., U.S.A.), 2,5-diphenyloxazole (PPO) and 1,4-bis(5-phenyloxazol 2-yl)-benzene (POPOP) from Packard Instrument Co. (Downers Grove, Ill., U.S.A.), millipore filters (HAWP 6025) from Millipore Filter Corp. (Bedford, Mass. U.S.A.), Sephadex G-25 and DEAE-Sephadex A-50 from Pharmacia (Uppsala, Sweden), and DEAE-cellulose from Serva (Heidelberg, G.F.R.). Tetracycline hydrochloride was purchased from Roussel UCLAF (France), puromycin dihydrochloride from Nutritional Biochemicals Co. (Cleveland, Ohio, U.S.A.), edeine from Polfa (Tarchomin, Poland), [CH_3-^3H]methionine (sp.act. 600 mCi and 4.5 C/mmol) from CEA (France) and [U-^{14}C]alanine (sp.act. 79.6 mCi/mmol) from the Institute of Radioisotopes (Praha, Czechoslovakia).

Methods. Ribosomes and crude initiation factors were prepared from *E. coli* MRE 600 as described by Zagórski *et al.* (1972).

Growth and isolation of phage f2, preparation of phage RNA and examination of its homogeneity were performed as described by Zagórski *et al.* (1972). About 70% of f2 RNA was found to sediment in the 27 S region.

Factor EF T ($T_u + T_s$) was prepared from the 40 - 65% ammonium sulphate fraction of the 105 000 g supernatant of a crude *E. coli* extract as described by Zagórska *et al.* (1971).

As a source of aminoacyl-tRNA synthetase, the 105 000 g supernatant purified by chromatography on DEAE-cellulose column, was used. f[³H]Met-tRNA was prepared by charging *E. coli* tRNA with [³H]methionine, formylation, discharging of non-formylated Met-tRNA and final purification of f[³H]Met-tRNA on Sephadex G-25 according to Lelong *et al.* (1970).

[¹⁴C]Ala-tRNA was prepared by charging *E. coli* tRNA with [¹⁴C]alanine in a mixture (20 ml) containing: 0.1 M-tris-HCl, pH 7.5, 0.01 M-$MgCl_2$, 0.05 M-KCl, 0.006 M-ATP, 13.2 mg of protein from the 105 000 g supernatant purified on DEAE-cellulose, 0.4 μmol of [¹⁴C]alanine and 70 mg *E. coli* tRNA. The [¹⁴C]Ala-tRNA was purified by the phenol method followed by gel filtration through a Sephadex G-25 column.

The binding of f[³H]Met-tRNA to ribosomes was performed in a medium (50 μl) containing 50 mM-tris-HCl, pH 7.5, 80 mM-NH_4Cl, 12 mM-2-mercapto-ethanol, 4.6 mM-magnesium acetate, 1 mM-GTP or, where indicated, GMP-PCP, 1.1 A_{260} units of f[³H]Met-tRNA (296 counts×min⁻¹×pmol⁻¹), 1.5 A_{260} units of ribosomes washed with 1 M-NH_4Cl, 30 μg of crude initiation factors and 70 μg of f2 RNA.

The binding of [¹⁴C]Ala-tRNA to ribosomes was performed in the same medium containing in addition 50 μg of factor EF T and 1.1 A_{260} units of [¹⁴C]Ala-tRNA (74 counts×min⁻¹×pmol⁻¹). The activity of f[³H]Met-tRNA used in double-labelling experiments was 3640 counts×min⁻¹×pmol⁻¹.

Incubation was performed for 20 min at 25°C and the amount of AA-tRNA bound to ribosomes was measured by the millipore filter technique (Nirenberg & Leder, 1964).

Radioactivity was determined in the Packard Tri-Carb liquid scintillation spectrometer in PPO and POPOP solution in toluene.

To investigate the reaction of puromycin with fMet-tRNA bound to the ribosome-tRNA complex, 40 μg of puromycin was added to the incubation mixture after completion of the binding assay, and the mixture was left for 5 or 30 min at 25°C. The results were the same regardless of the time of incubation. Formylmethionyl-puromycin was extracted with ethylacetate at pH 5 according to Leder & Bursztyn (1966) and radioactivity was determined in Bray's solution (Bray, 1960).

All values given in Results have been corrected by subtracting background without f2 RNA.

RESULTS

General reaction conditions

Binding of fMet-tRNA to ribosomes. The first set of experiments was designed to establish the effect of Mg^{2+} concentration on the binding of fMet-tRNA to ribosomes in a system directed by f2 RNA, and the effect of initiation factors on the binding reaction. The maximum fMet-tRNA binding to ribosomes was observed at 4.6 mM-Mg^{2+} (Fig. 1), and this concentration was used in all subsequent experiments. The binding of fMet-tRNA to ribosomes in the system under study depends on the presence of initiation factors (Table 1). Low optimal Mg^{2+} concentration as well as dependence on initiation factors indicate that fMet-tRNA binding to ribosomes reflects the process of specific initiation (Clark *et al.*, 1970).
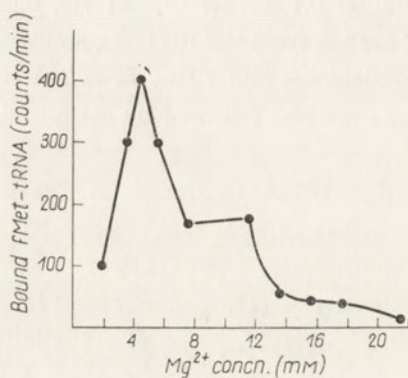

Fig. 1. Effect of Mg^{2+} concentration on the binding of f[^{3}H]Met-tRNA to ribosomes in the presence of f2 RNA. The binding assay was performed as described in Methods; temperature 25°C, time 20 min

Table 1

Effect of initiation factors on the binding of f[^{3}H]Met-tRNA to ribosomes in the presence of f2 RNA

The binding assay was performed as described in Methods.

System	fMet-tRNA bound to ribosomes (pmol)	
	without f2 RNA	with f2 RNA
Complete	0.670	2.280
Initiation factors omitted	0.172	0.125

The effect of temperature and time of incubation on the formation of initiation complex is shown in Fig. 2. The amount of fMet-tRNA bound to ribosomes increased with temperature and maximum binding was reached after 5 min of incubation at 37°C. However, the initiation complex was unstable at this temperature and was decomposed on prolonged incubation. At 25°C the initiation complex was formed

with lower yield but its stability was greater; therefore all subsequent experiments were carried out at 25°C. The highest binding of fMet-tRNA to ribosomes was observed when the incubation mixture contained 65 pmol of f2 RNA and 36.5 pmol of ribosomes.
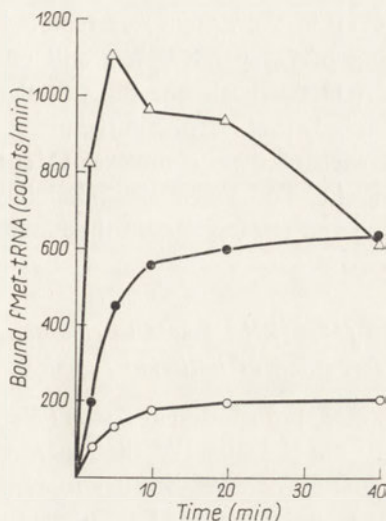

Fig. 2. Effect of time and temperature on binding of f[^{3}H]Met-tRNA to ribosomes. The mixtures (250 μl) were incubated at 15° (○), 25° (●), and 37°C (△). At the indicated times 50 μl samples were withdrawn and analysed as described in Methods.

Binding of Ala-tRNA to ribosomes. In the coat protein cistron of phage f2 the initiation codon is followed by a codon specific for alanine (Steitz, 1969). Hence, in an *in vitro* system directed by f2 RNA and synthesizing mainly the coat protein, the first AA-tRNA bound to ribosomes after fMet-tRNA is Ala-tRNA. As shown in Table 2, Ala-tRNA and fMet-tRNA were bound to ribosomes at an equimolar ratio. The binding of Ala-tRNA depended on previous binding of fMet-tRNA. When fMet-tRNA or the initiation factors were omitted from the system, Ala-

Table 2

f2 RNA directed binding of f[^{3}H]Met-tRNA and [^{14}C]Ala-tRNA to ribosomes

The binding assay was performed as described in Methods.

System (Reaction components)	fMet-tRNA bound to ribosomes (pmol)	Ala-tRNA bound to ribosomes (pmol)
Complete	2.56	2.70
EF T omitted	3.34	3.44
IF omitted	0.00	0.00
fMet-tRNA omitted	0.00	0.00
GTP omitted	0.20	—
GTP omitted, GMP-PCP added	2.35	0.19

tRNA was not bound to ribosomes. The binding of Ala-tRNA in the system under study was not stimulated by addition of purified factor EF T. This was probably due to the presence of factor EF T in the crude preparation of initiation factors. The binding of fMet-tRNA and Ala-tRNA to ribosomes depended on the presence of GTP. When GTP in the system was replaced by GMP-PCP, its non-splittable analogue, binding of fMet-tRNA was still observed but that of Ala-tRNA no longer occurred. On this basis one can postulate the occurrence of two forms of initiation complex, only that existing in the presence of GTP being capable of binding Ala-tRNA. In each of these complexes fMet-tRNA may be bound to the ribosome at a different site. To confirm this possibility we tested the mode of action of puromycin, edeine and tetracycline on the binding of AA-tRNA to ribosomes.

Reaction of puromycin with fMet-tRNA bound to ribosomes and effect of edeine on the formation of initiation complex

According to Watson's model of ribosomal action (Watson, 1964), in our system fMet-tRNA should occupy the ribosomal P site whereas Ala-tRNA, the A site.

The reaction of the initiation complex with puromycin showed that all fMet-tRNA bound to ribosomes in the presence of GTP reacted freely with puromycin. In a system in which 0.95 pmol of fMet-tRNA was bound to ribosomes, 4.4 pmol of formylmethionine were recovered in the product formed in the presence of puromycin. The excess of formylmethionine recovered from the product obtained with puromycin may have resulted from antibiotic-induced changes in reaction equilibrium favouring binding to ribosome of additional portions of fMet-tRNA which subsequently reacted with puromycin. The high activity in the presence of puromycin indicated that in our system supplemented with GTP all fMet-tRNA recovered from the initiation complex occupied the P site.

To prove this assumption we investigated the effect of edeine on fMet-tRNA binding to ribosomes in the presence of GTP (Fig. 3). It was found that in a system directed by f2 RNA, edeine blocked the binding of fMet-tRNA to ribosomes. According to Szer & Kuryło-Borowska (1970) edeine inhibits the attachment of AA-tRNA to the P site. Hence, the observed effect can be taken as additional evidence for the P site binding of fMet-tRNA in an initiation complex.

The influence of tetracycline on the binding of fMet-tRNA and Ala-tRNA to ribosomes

fMet-tRNA bound to the P site could have entered this site indirectly *via* the A site. To test this possibility the effect of tetracycline on the binding of initiator tRNA was investigated.

In the system under study binding of Ala-tRNA reflects the attachment of AA-tRNA to the A site. As shown in Table 3, the binding of Ala-tRNA was strongly inhibited by tetracycline. By contrast, the antibiotic did not, even at high concentrations, inhibit the binding of fMet-tRNA and at low concentrations it even stimulated it. These results indicate that fMet-tRNA does not even temporarily enter the A site.
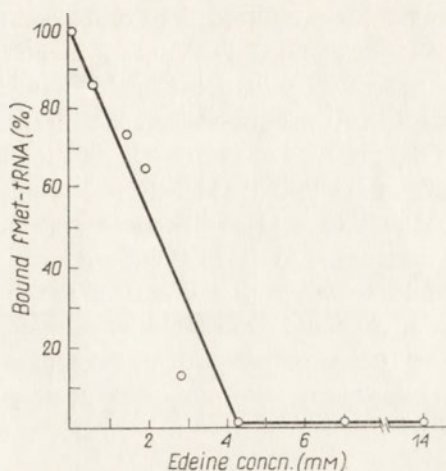
Fig. 3. Effect of edeine on the binding of f[^{3}H]Met-tRNA to ribosomes. The binding assay was performed as described in Methods.

However, the experiment does not exclude an intermediate location of initiator tRNA prior to its attachment to the P site. This hypothetical structural intermediate can be regarded as an f2 RNA-ribosome-fMet-tRNA-IF 2-GTP complex and it probably exists at the first stages of initiation complex formation, prior to hydrolysis of GTP. To elucidate the reality of this concept we studied the binding of fMet-tRNA in the presence of GMP-PCP.

Table 3

Effect of tetracycline on the binding of f[^{3}H]Met-tRNA and [^{14}C]Ala-tRNA to ribosomes in the presence of f2 RNA

The binding assay was performed in the presence of tetracycline at indicated concentrations as described in Methods.

Tetracycline concn.	fMet-tRNA bound to ribosomes		Ala-tRNA bound to ribosomes	
	counts/min	%	counts/min	%
None	6 591	100	169	100
10^{-5} M	10 440	159	123	73
5×10^{-5} M	9 676	146	105	62
10^{-4} M	10 953	167	77	45
5×10^{-4} M	8 027	122	31	19
10^{-3} M	6 909	105	17	10

Binding of fMet-tRNA to ribosomes in the presence of GMP-PCP

As shown in Table 2, the same amount of fMet-tRNA bound to ribosomes in the presence of GTP as with GMP-PCP. The binding of fMet-tRNA in the presence of GMP-PCP was found to be insensitive to tetracycline similarly as the GTP-

dependent binding (Table 3). It can therefore be concluded that fMet-tRNA bound to ribosomes in the presence of GMP-PCP does not enter the A site. The effect of edeine on the formation of the initiation complex was identical in the presence of either GTP or GMP-PCP. At 4.2 mM-edeine concentration the binding of fMet-tRNA to ribosomes in the presence of GMP-PCP was totally inhibited. This indicates that in the presence of GMP-PCP fMet-tRNA occupies a site resembling the P site. However, in contrast to a GTP-dependent system, fMet-tRNA attached to the ribosome in the presence of GMP-PCP did not react with puromycin (Table 4) and consequently could not serve as a donor of the formylmethionyl residue. The reactivity with puromycin could be restored by addition of GTP to the system. These data suggest the existence of two forms of complex between the ribosome and initiator tRNA: (1) unreactive with puromycin, detectable in the presence of GMP-PCP and (2) reactive, generated from the unreactive one by GTP.

Table 4
Effect of GMP-PCP and GTP on the reactivity of f[³H]Met-tRNA with puromycin

The binding assay and reaction with puromycin were performed as described in Methods except that in expt. 2 after 10 min of incubation in the presence of GMP-PCP, GTP was added to 1 mM concentration and incubation was continued for another 10 min.

Expt. no.	Initiation complex formed in the presence of	fMet-tRNA bound to ribosomes (pmol)	fMet-puromycin product (pmol)
1	GMP-PCP	1.05	0.068
2	GMP-PCP, GTP	1.10	2.300

Tentative localization of puromycin and tetracycline sensitive sites on the ribosome

When examining the reaction of puromycin with a ribosomal complex containing both fMet-tRNA and Ala-tRNA we observed a low yield of the puromycin derivative (Table 5). The low reactivity of puromycin under these conditions may be

Table 5
Reaction of puromycin with a ribosomal complex containing bound f[³H]Met-tRNA and [¹⁴C]Ala-tRNA

Reaction conditions as described in Methods.

tRNA bound to ribosomes		Product with puromycin			
		containing fMet		containing Ala	
fMet-tRNA (pmol)	Ala-tRNA (pmol)	pmol	%	pmol	%
1.72	1.80	0.55	32	0.12	7

attributed to the presence of Ala-tRNA at the A site and its preventing the attachment of puromycin. This would suggest that the site of puromycin attachment and the A site partially overlap, probably in a region of the 50 S subunit close to peptidyl transferase.

The experiments previously described suggest that tetracycline reacts with the 50 S subunit in the region of the A site (Zagórska et al., 1971). When the puromycin reaction was followed in the presence of tetracycline, it was observed (results not shown here) that tetracycline did not inhibit the initation complex reactivity with puromycin. This would indicate that tetracycline affects a region of the A site different from the region affected by puromycin.

DISCUSSION

The results presented in this paper show that in a GTP-dependent system directed by a natural template fMet-tRNA is bound to the ribosomal P site without preliminary interaction with the A site. This supports a similar conclusion drawn from a study on the action of tetracycline on the poly AUG-stimulated binding of initiator tRNA (Zagórska et al., 1971) and is in agreement with the recent experiments of Thach & Thach (1971) and Kuechler (1971).

In the presence of GMP-PCP the f2 RNA-directed binding of initiator tRNA is insensitive to tetracycline and strongly inhibited by edeine, resembling in this respect the GTP-dependent attachment of fMet-tRNA. However, in contrast to the situation observed with a system supplemented with GTP, the initiator tRNA bound to the ribosome in the presence of GMP-PCP does not react with puromycin.

The attachment of initiator tRNA in the presence of GTP or GMP-PCP at the P site has recently been demonstrated by Benne & Voorma (1972). Lack of puromycin reactivity observed in the system was attributed by these authors to the presence of initiation factor IF 2 firmly attached to GMP-PCP and to the ribosome. This form of the initiation complex, from which the initiation factor has not been removed, may correspond to the previously proposed pre-initiation complex adopting transient unreactive conformation before correct positioning of fMet-tRNA (Zagórska et al., 1971), as well as to a complex in which initiator tRNA is bound to the "pre P" site (Thach & Thach, 1971).

Our experiments show that the initiation complex formed in the presence of GMP-PCP, which does not react with puromycin, can be converted to a reactive form by adding GTP (see Table 5). The GTP-induced conversion of the pre-initiation complex to a puromycin reactive form may be interpreted by exchange of GMP-PCP for GTP, followed by hydrolysis of the energy rich bond and concomitant release of the initiation factor from the ribosome (Thach & Thach, 1971).

It may thus be proposed that before fMet-tRNA partaking in the initiation complex acts as a donor of the formylmethionyl group, it is bound to the ribosome in a transient complex containing GTP and IF 2. Hydrolysis of GTP and dissociation of IF 2 from the ribosome leads to the formation of the proper initiation complex necessary for starting the elongation cycle.

The difference in positioning of initiator tRNA in the two types of complexes can be attributed to changes occurring in the 50 S ribosomal subunit. The two types of complexes can be distinguished with puromycin, which reacts with the 50 S subunit (Monro, 1967), but not with edeine, which reacts with the 30 S subunit (Szer & Kuryło-Borowska, 1970).

It should be emphasized that the pre-initiation complex, unreactive with puromycin, cannot bind Ala-tRNA (see Table 2). This can be explained by assuming that the conformational changes induced by the presence of initiation factor IF 2 in the pre-initiation complex affect the A site region. The results presented are in agreement with the postulated active role of initiation factors in selection of initiation sites on the ribosome and in changing the ribosomal conformation during initiation (Springer *et al.*, 1971).

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MIEJSCE PRZYŁĄCZANIA FORMYLOMETIONYLO-tRNA DO RYBOSOMÓW *ESCHERICHIA COLI* W OBECNOŚCI RNA FAGA f2

Streszczenie

1. Tetracyklina w stężeniu $0,5 - 1 \times 10^{-4}$ M hamuje przyłączanie Ala-tRNA do rybosomów w obecności RNA faga f2, nie wpływa jednak na przyłączanie fMet-tRNA.

2. Edeina hamuje zależne od RNA faga f2 tworzenie kompleksu inicjującego.

3. Gdy GTP zastąpiono GMP-PCP, przyłączanie fMet-tRNA do rybosomów również nie było hamowane przez tetracyklinę. Przyłączony w tych warunkach inicjatorowy tRNA nie może być jednak donorem reszty formylometionylowej.

4. Wyniki sugerują, że fMet-tRNA, zanim osiągnie właściwą pozycję w miejscu P, przyjmuje w obrębie tego miejsca przejściową konformację, która nie jest zdolna do reakcji z puromycyną i do zainicjowania syntezy łańcucha peptydowego.

RECENZJE KSIĄŻEK

THE ROLE OF ADENYL CYCLASE AND CYCLIC 3′,5′-AMP IN BIOLOGICAL SYSTEMS.
A colloquium sponsored by the John E. Fogarty International Center for advanced Study in the
Health Sciences. National Institutes of Health, Bethesda, Maryland. Fogarty International
Center Proceedings no. 4; pp. 295.

Konferencja zorganizowana w 15 rocznicę zapoczątkowania przez dr E. W. Sutherlanda i jego
współpracowników badań nad cyklazą adenilową i cyklicznym AMP odbyła się 17-19 listopada
1969 roku.

Badania Sutherlanda i współpracowników nad działaniem adrenaliny i glukagonu na glikolizę
doprowadziły do odkrycia cyklicznego AMP i cyklazy adenilowej. Były to pierwsze doświadczenia,
w których bezspornie wykazano działanie hormonu w bezkomórkowych preparatach. Obaliło
to pogląd, że nienaruszona struktura komórki jest konieczna dla działania hormonu, a równo-
cześnie potwierdziło przypuszczenie, że hormon może działać na enzymy. Badania te doprowadziły
do przedstawionej przez Sutherlanda i Ralla koncepcji o roli cyklicznego AMP jako wewnątrz-
komórkowego mediatora działania hormonu.

Od tego czasu badania nad cyklicznym AMP i cyklazą adenilową tak się rozwinęły i objęły
tak rozległe dziedziny biologii, że nie sposób było zorganizować konferencję obejmującą wszystkich
zainteresowanych tym zagadnieniem badaczy. W kolokwium wzięło udział 45 naukowców, którzy
reprezentowali różne kierunki badań. Wielu z nich było lub jest współpracownikami dr Suther-
landa, który niestety z powodu choroby nie mógł wziąć udziału w obradach.

Na konferencji wygłoszono 13 referatów, w których prelegenci przedstawili głównie własne
badania. Po każdym referacie była ożywiona i bardzo ciekawa dyskusja. O zagadnieniach dysku-
towanych na konferencji może dać pojęcie zestawienie tytułów referatów:

Własności cyklazy adenilowej u ssaków (Theodore W. Rall)
Własności bakteryjnej cyklazy adenilowej (Fritz Lipmann)
Działanie hormonów na cyklazę adenilową (Reginald W. Butcher)
Hormony, receptory i aktywność cyklazy adenilowej w komórkach ssaków (Martin Rodbell)
Istota receptorów oraz związek pomiędzy strukturą a aktywnością hormonów polipeptydowych
    (Robert Schwyzer)
Cykliczny AMP jako czynnik regulujący w metabolizmie cukrowców i lipidów (Edwin G. Krebs)
Rola cyklicznego AMP w glukoneogenezie wątrobowej (Charles R. Park)
Cykliczny AMP jako czynnik regulujący w układach aktywnego transportu (François Morel)
Rola cyklicznego AMP w procesach wydzielniczych (Michael Schramm)
Rola cyklicznego AMP w indukcji enzymów u *E. coli* (Ira Pastan)
Cykliczny AMP a kataboliczna represja u *E. coli* (Agnes Ullmann)
Cykliczny AMP w różnicowaniu komórek u śluzowców (John Tyler Bonner)
Regulacja poziomu cyklicznego AMP (Theodore W. Rall).

Na zakończenie należy przypomnieć, że dr Earl W. Sutherland otrzymał w roku 1970 nagrodę
Alberta Laskera w zakresie podstawowych badań medycznych, a w roku 1971 nagrodę Nobla.
Jak piszą we wstępie książki organizatorzy konferencji, dr Martin Rodbell i dr Peter Condliffe,
,,znaczenie odkrycia cyklicznego AMP dla nauk biologicznych można porównać z odkryciem ATP;
cykliczny AMP ma równie podstawowe znaczenie dla bio-regulacji, jak ATP dla bio-energetyki''.

Irena Mochnacka

[R1]

KURZGEFASSTES LEHRBUCH DER PHYSIOLOGIE (W. D. Keidel, ed.). G. Thieme Verlag, Stuttgart 1970. II wydanie, str. 524, cena 39.80 DM.

Podręcznik stanowi bardzo dobry przykład nowoczesnego i w miarę zwięzłego podręcznika akademickiego. Pozytywną jego cechą jest szerokie uwzględnienie, a nawet wybicie na pierwszy plan, problemy regulacji fizjologicznych i przekazywanie studentom w sposób nowoczesny myślenia fizjologicznego w kategoriach związków i zależności regulacyjnych całego organizmu. Bardzo szeroko uwzględniona jest ogólna teoria regulacji (biocybernetyka), stanowiąca rozdział wprowadzający do podręcznika (D. Keidel). Zastosowanie teorii informacji w fizjologii narządów zmysłów i układu nerwowego ujęte jest w osobny obszerny rozdział (16), wymagający wszakże pewnego przygotowania z zakresu matematyki.

Podręcznik uwzględnia bardzo szeroko elementy biofizyki, zwłaszcza w rozdziałach dotyczących fizjologii naczyń krwionośnych (6) i serca (5).

Bardzo przystępnie, a zarazem nowocześnie potraktowana jest elektrofizjologia komórki i włókna nerwowego (rozdział 14). Korzystną innowacją jest wprowadzenie osobnego, acz bardzo zwięzłego, rozdziału o fizjologii pracy (J. Stegemann). Szczupłe ramy podręcznika i wyeksponowanie elementów biofizyki spowodowały pewną nadmierną zwięzłość niektórych rozdziałów (hormony), lub pominięcie szeregu szczegółowych informacji (np. brak omówienia prostaglandyn).

Podręcznik zaopatrzony jest w bardzo dobre dydaktycznie ilustracje, upraszczające niekiedy złożone mechanizmy ale ułatwiające percepcję materiału. W sumie jest to bardzo wartościowa książka, odbiegająca korzystnie nowoczesnością ujęcia od wielu tradycyjnych opisowych podręczników fizjologii.

Andrzej Trzebski

Emil Ginter, THE ROLE OF ASCORBIC ACID IN CHOLESTEROL METABOLISM. Rola kwasu askorbinowego w przemianie cholesterolu. Seria Ogólnej i Specjalnej Biologii Słowackiej Akademii Nauk, XVI/6. Bratysława 1970; str. 100.

Książeczka jest podsumowaniem dziesięcioletniej pracy grupy badawczej Oddziału Biochemii Instytutu Badań Żywienia w Bratysławie. Publikacja ma charakter monografii, jest zwarta i zawiera dużą ilość uporządkowanych informacji oraz ich krytyczny przegląd. Omówienie własnych prac grupy autora zajmuje połowę tekstu.

Autor omawia najpierw funkcje witaminy C w syntezie kolagenu, w przemianach tyrozyny, w syntezie noradrenaliny, w hydroksylacji hormonów sterydowych, w biosyntezie serotoniny, w transporcie żelaza i elektronów, oraz zaburzenia przemiany tłuszczowców w ostrym niedoborze tej witaminy i jej rolę w miażdżycy.

Następnie autor przedstawia wprowadzony przez siebie i grupę współpracowników nowy model badania na świnkach morskich przewlekłego niedoboru kwasu askorbinowego, podobnego do naturalnego niedoboru tego związku u ludzi. Również na świnkach morskich opracowano model doświadczalnej hipercholesterolemii i miażdżycy, co umożliwiło długotrwałą obserwację wpływu niedoboru witaminy C na patomorfologię naczyń.

W przewlekłym niedoborze kwasu askorbinowego — w przeciwieństwie do niedoboru ostrego — występuje znaczące nagromadzanie się cholesterolu w wątrobie oraz wzrost w surowicy krwi β-lipoproteidów. Stwierdzono m.in., że istnieje odwrotnie proporcjonalna zależność między stężeniem cholesterolu a kwasu askorbinowego.

Zmiany patomorfologiczne narządów świnek morskich karmionych aterogenną dietą cholesterolową były bardziej nasilone przy niedoborze witaminy C niż bez niedoboru tej witaminy.

Badania mechanizmu zwiększonego nagromadzania się cholesterolu przy niedoborze witaminy C wykazały, że nie wynika ono ze zwiększonej syntezy cholesterolu ani ze zwiększonego wchłaniania z przewodu pokarmowego, a jest skutkiem upośledzenia przemiany tego związku do kwasów żółciowych. Autor wiąże to z przypuszczalnym wpływem kwasu askorbinowego na a-hydroksylację C_7 i C_{12} układu cyklopentanoperhydrofenantrowego.

Oprócz badań na zwierzętach, przedstawiono badania typu epidemiologicznego, dotyczące związku między sezonowymi, związanymi z porą roku wahaniami cholesterolemii u ludzi, a nasyceniem lub utajonym brakiem witaminy C, uwarunkowanym okresowymi zmianami jej podaży.

Z bogatej cytowanej literatury (316 pozycji) jest 26 pozycji autora monografii.

Leszek Tomaszewski

H. G. Schlegel, ALLGEMEINE MIKROBIOLOGIE. Zweite, überarbeitete und erweiterte Auflage. G. Thieme Verlag. Stuttgart 1972; str. XIII + 461, cena 16,80 DM.

Wydawca słusznie charakteryzuje tę pracę w następujących słowach: ,,Książka przeznaczona jest dla szerokiego kręgu czytelników. Ma ona służyć przede wszystkim mikrobiologom jako pełny przegląd mikrobiologii ogólnej. W książce położono główny nacisk na fizjologię metabolizmu, uwzględniono przy tym współczesne dziedziny genetyki biochemicznej, komórkowe regulacje metabolizmu, wyniki badań nad mutantami metabolicznymi. Książka ma także aspekty praktyczne, dlatego też może także służyć dla studentów jako źródło informacji, jak również może być wprowadzeniem ułatwiającym zapoznanie się z biologią molekularną".

Recenzja pierwszego wydania z roku 1969 została opublikowana w Acta Biochimica Polonica (T. Korzybski, Acta Biochim. Polon. 1969, **16**, R6). Nowe wydanie zostało w wielu miejscach poprawione i rozszerzone. Dodano rozdział o pigmentach bakteryjnych i grzybowych, umieszczono nowe akapity o budowie wirusów, lizogenii, o modyfikacjach DNA fagowego, o wirusowej teorii powstawania raka, o mechanizmie transportu substancji z podłoża do wnętrza komórki, o wpływie antybiotyków na biosyntezę białka, o sposobach konserwacji środków spożywczych i innych materiałów biologicznych, o powstawaniu antybiotyków, o toksynach grzybowych (aflatoksyna) i o procesach zachodzących w żołądkach przeżuwaczy. Strona ilustracyjna nowego wydania jest bogatsza, zawiera większą liczbę ilustracji, a poprzednie ilustracje zostały w wielu przypadkach zmienione na korzyść. Typografia wzorów chemicznych została poprawiona. W wykazie literatury podano nowsze opracowania uwzględniające rok 1970. Nieliczne zauważone błędy (wzory penicyliny na str. 278, niepotrzebna cyfra 5 przy nazwie metyloglukozaminy na str. 283) nie odgrywają istotnej roli w pozytywnej ocenie tego zwięzłego, jasno napisanego, podręcznego dziełka, mogącego z powodzeniem służyć do celów, które wymieniono na początku tej recenzji.

Tadeusz Korzybski

PROTIDES OF THE BIOLOGICAL FLUIDS. Proceedings of the Nineteenth Colloquium, Bruges, 1971 (H. Peeters, ed.). Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1972; str. 589, cena $ 16.0.

Książka stanowi zbiór materiałów 19-tej międzynarodowej konferencji poświęconej płynom biologicznym, która odbyła się w Bruges (Belgia) w 1971 r. Główne tematy konferencji dotyczyły lipoprotein, hydrodynamicznych właściwości białek, katabolizmu białek oraz niektórych nowych metod. Niemal wszystkie artykuły przedstawiają wyniki badań własnych.

Najwięcej uwagi poświęcono lipoproteinom (Sekcja A). W ponad 60 artykułach poruszane są takie zagadnienia, jak: skład, struktura i funkcja, synteza i genetyka lipoprotein, a ponadto patologia tych białek. W Sekcji B (11 artykułów) wydzielono problemy związane z hydrodynamicznymi własnościami białek. Znalazły się tam artykuły opisujące metody mierzenia hydratacji i momentów dipolowych makromolekuł naturalnych i syntetycznych, a także wykorzystania techniki fluorescencyjnej do badania struktury polipeptydów. Sekcja C zawiera artykuły (15) dotyczące rozpadu szeregu białek osocza krwi, a szczególnie immunoglobulin. Na ostatnią część książki (Sekcja D) składają się artykuły (15) przedstawiające nowe metody i techniki rozdziału i ilościowego oznaczania różnych białek bądź frakcji białkowych osocza. Większa część materiału dotyczy metod immunologicznych.

Wydawanie zbiorów artykułów z sympozjów czy konferencji tematycznych jest celowe i z tego względu prezentowana książka jest wartościową pozycją. Szerszy ogół czytelników nie znajdzie, niestety, obszerniejszych artykułów przeglądowych. Brak wówniez materiałów z dyskusji, co należy uznać za mankament omawianej książki. Z uwagi na szeroki zakres omawianych zagadnień książka winna zainteresować nie tylko specjalistów zajmujących się lipoproteinami, ale i szeroki ogół bio-chemików. Bogata bibliografia podana przy każdym artykule oraz staranna edycja podnoszą walory książki.

Ryszard Niemiro

ACTA BIOCHIMICA POLONICA

PRENUMERATA KRAJOWA

PRENUMERATA ZAGRANICZNA