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MODIFICATION OF D-LACTATE DEHYDROGENASE FROM ANAEROBICALLY GROWN YEAST *

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1. The enzyme was modified by treatment with cysteine in the presence of urea and by subsequent oxidation with 2,6-dichlorophenol indophenol. 2. The modified preparation lost the ability to reduce ferricyanide, whereas its activity towards indophenol remained unaffected. The modification was reversible. 3. A possible mechanism for the reactions occurring during the modification is suggested.

Studies on the structure and function of the active site of enzymes form an essential stage in elucidating the mechanisms of their biological activity [4]. Although, for the most part, these studies concern one enzyme only, more information can be obtained by studying a group of enzymes possessing similar catalytic properties. Lactic dehydrogenases form one of the groups which have attracted considerable attention. However, owing to difficulties in obtaining pure preparations, the individual enzymes are usually studied by kinetic and not analytical methods. D(-)Lactate dehydrogenase isolated from anaerobically grown yeast [7] has been extensively studied so far only by kinetic methods [1, 6]. In the present work, the chemical modification of the active site of D-lactate dehydrogenase was undertaken as an attempt to study properties of lactate dehydrogenases.

MATERIALS AND METHODS

Reagents. Sodium D-lactate was obtained from calcium D-lactate produced by CalBiochem (Los Angeles, U.S.A.). L-Cysteine was obtained from Fluka (Switzerland) or CalBiochem (Los Angeles, U.S.A.), and Sephadex G-50 from Pharmacia (Uppsala, Sweden). Sodium phosphates, ammonium sulphate, 2,6-dichlorophenol indophenol (indophenol), ferricyanide and urea were reagent grade products of

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Fabryka Odczynników Chemicznych, Gliwice, Poland. The urea was purified by crystallization from 60% ethanol.

Enzyme. The purified preparation of D(-)lactate dehydrogenase was obtained from anaerobically grown yeast [3, 6].

Determination of enzymic activity. This was performed by measuring the rate of reduction of ferricyanide at 420 mμ and of 2,6-dichlorophenol indophenol at 600 mμ in SF-5 spectrophotometer (U.S.S.R.). The reaction mixture contained in a final volume of 3 ml.: 2 μmoles of ferricyanide or 0.33 μmole of indophenol, 25 μmoles of sodium D-lactate, 200 μmoles of Na-phosphate buffer, pH 7, and 0.1 ml. of enzyme solution containing 0.1-0.5 mg. protein. Extinction was measured during 1 min. at 10 sec. intervals. From the data obtained the average change in extinction was calculated per 1 min. The specific activity was expressed as mμmoles of the electron acceptor reduced/min./mg. protein.

Protein was determined by the turbidimetric method [5] in the Coleman B-14 spectrophotometer.

Method of enzyme modification. The modification consisted of: (1), treatment of the enzyme with cysteine in the presence of urea, and (2), after removal of urea and excess cysteine, oxidation of the enzyme with 2,6-dichlorophenol indophenol. The enzyme preparation containing about 60 mg. of protein in a volume of 0.5 ml., was incubated for 5 min. at 0° with an equal volume of 0.5 M-cysteine solution, pH 9.6, in the presence of 3 M-urea. The solution was, then, adjusted to pH 7 with 0.5 M-KH_2PO_4 and protein was precipitated by adding ammonium sulphate to 0.6 saturation. The sediment was centrifuged in a cooled Servall type centrifuge at 14 000 *g* for 5 min., then washed with 0.6 sat. solution of ammonium sulphate and centrifuged as above. The protein was dissolved in 0.5 ml. of 0.2 M-phosphate buffer, pH 8, the undissolved sediment being discarded. The dissolved protein was oxidized with indophenol at a final concentration of 7.5 mM, and applied to a column (10 × 100 mm.) of Sephadex G-50, which had been washed with 0.2 M-phosphate buffer, pH 7.2. In the gel-filtrate the content of protein and the activity towards ferricyanide and indophenol were determined.

In some experiments the first step was altered: the enzyme was treated with cysteine alone, or in the presence of urea and 25 mM-sodium lactate, or the enzyme was oxidized prior to the cysteine-urea treatment. In the latter case, 0.5 ml. of the enzyme was incubated for 5 min. with 0.1 ml. of 3.3 mM-indophenol (the final concentration being 0.55 mM). Then cysteine solution and urea were added, the mixture incubated for 5 min. and treated as above.

As control, an untreated native enzyme preparation, precipitated by 0.6 ammonium sulphate saturation was used.

Reversal of the modification. The modified enzyme preparation containing about 20 mg. of protein in 0.5 ml. of 0.2 M-phosphate buffer, pH 8, was incubated for 10 min. with an equal volume of 0.25 M-cysteine solution, pH 9.6, at room temperature, then applied to a Sephadex G-50 column prepared as above. Protein in the ge-filtrate was precipitated by ammonium sulphate at 0.6 saturation, centrifuged, dilssolved, and the protein content and the activities were assayed.

RESULTS AND DISCUSSION

The effects of modification of D-lactate dehydrogenase from anaerobically grown yeast, are presented in Table 1. The enzyme incubated with cysteine in the presence of urea became inactive towards ferricyanide. In the absence of urea, the inactivation was but partial. In either case the activity towards indophenol was retained. If the enzyme preparation was treated with cysteine after being oxidized, no modification was observed. However, a decrease appeared in specific activity towards both acceptors, amounting to 25% of the value for the native enzyme. This decrease can be explained by inactivation of the enzyme by indophenol at the concentration applied.

Table 1

The effect of cysteine on the activity of D-*lactate dehydrogenase from yeast grown anaerobically*

Details of the treatment and activity determinations are described under Methods. The specific activity is expressed as mμmoles of reduced acceptor/min./mg. protein

Treatment of the enzyme	Protein		Activity			Relation of activity: ferricyanide to indophenol
	total (mg.)	recovery after treatment (%)	specific		total	
			Ferri-cyanide	Indo-phenol	Indo-phenol	
None (native)	66	100	250	230	15 180	1.08
Cysteine	34	55	190	420	14 280	0.45
Cysteine and 3 M-urea	23	35	0	260	5 980	0
Oxidized with indophenol, then cysteine and 3 M-urea	14	26	60	60	840	1
Cysteine, 3 M-urea and lactate	21	32	280	325	6 825	0.86

The modification caused a decrease in the content of soluble protein in the enzyme preparation. The recovery after modification in the absence of urea amounted to 55% (Table 1), and in the presence of urea to 35%, whereas after modification of the oxidized enzyme, it was only 25%. In control experiments, the presence of urea alone did not affect the solubility of protein.

The total activity towards indophenol of the enzyme modified in the presence of urea amounted to about 40% of the activity of the native enzyme, whereas after modification without urea the total activity was the same as that of the native enzyme. Simultaneously, in the latter case, a twofold increase in specific activity was observed (Table 1). This indicates that proteins other than the enzyme were removed. Total activities towards both acceptors in the enzyme modified after being oxidized, amounted only to 5%.

The applied modification of the enzyme probably concerns the active site. This supposition was confirmed by the observation that the enzyme treated with cysteine in the presence of D-lactate is able to reduce both acceptors.

The modified enzyme regains the ability to reduce ferricyanide after incubation with cysteine (Table 2). The reversal of the modification was also attempted by applying thioglycolate [2] which, however, was found to inactivate D-lactate dehydrogenase.

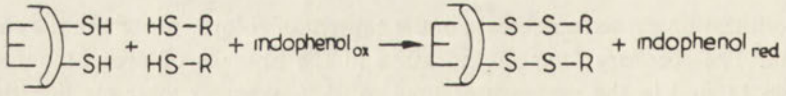
Table 2

The reversal of enzyme modification by cysteine

For details see Methods.

Enzyme	Activity (mμmoles of reduced acceptor/min./mg. protein)		Relation of activity: ferricyanide to indophenol
	Ferricyanide	Indophenol	
Native	90	90	1
Modified	0	80	0
After reversal of modification	40	50	0.8

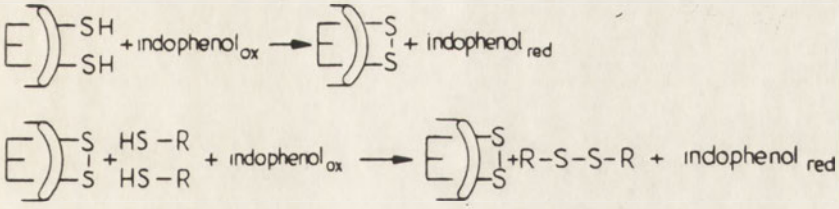
The susceptibility of D-lactate dehydrogenase to *p*-chloromercuribenzoate [6] indicates that thiol groups are essential for the activity of this enzyme. Moreover, the fact that ferricyanide, which reacts with thiol groups, can serve as an electron acceptor indicates the possibility that SH groups are located at the active site. Thus the described modification which blocks the reduction of ferricyanide would concern the SH groups, and it seems to consist in binding cysteine to a cysteine residue present at the active site of the enzyme, by disulphide linkage formed on oxidation with indophenol (Scheme 1).



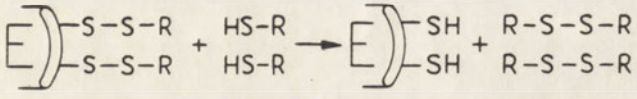
Scheme 1. The proposed mechanism of modification

When the native enzyme is oxidized prior to modification, internal S-S bonds are formed and therefore no modification by cysteine is obtained (Scheme 2).

The reversal by cysteine of the modification confirms the proposed mechanism. The reversal consists in breaking up the disulphide bonds formed during the modification between cysteine and the active site of the enzyme, and thus liberating the SH groups essential for the reduction of ferricyanide (Scheme 3).



Scheme 2. Modification of the oxidized form of the enzyme



Scheme 3. The reversal of modification

The results concerning the modification of the enzyme seem to indicate that different active sites are involved in the reduction of indophenol and ferricyanide. It seems probable that D-lactate dehydrogenase from yeast grown anaerobically has spatially separate mechanisms reacting with these electron acceptors.

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MODYFIKACJA DEHYDROGENAZY D-MLECZANOWEJ DROŻDŻY HODOWANYCH BEZTLENOWO

Streszczenie

1. Enzym modyfikowano przez poddanie go działaniu cysteiny w obecności mocznika a następnie utlenianie dwuchlorofenoloindofenolem.

2. Zmodyfikowany preparat tracił zdolność redukcji żelazicyjanku, podczas gdy aktywność wobec indofenolu pozostawała niezmieniona. Modyfikacja była odwracalna.

3. Podano przypuszczalny mechanizm reakcji zachodzących podczas modyfikacji.

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THE EFFECT OF pH ON THE BINDING OF NUCLEOTIDES BY ACTIN

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1. At pH values from 3.6 to 4.7 the complexes formed between actin and ATP are of similar nature as those formed by other proteins: the binding of ATP decreases with the increase of pH and increases with the increase of nucleotide concentration; heat denaturation has no influence on the binding ability. 2. Unlike other proteins, however, native actin shows in a neutral medium a specific ability to bind ATP (or ADP). The amount of bound nucleotide is greater than at isoelectric point (pH 4.7) and is independent of the nucleotide concentration; heat denatured actin loses the ability to bind nucleotides in neutral medium nearly completely. 3. The irreversible inactivation of actin proceeds faster at pH 3.6 than at pH 11.5. At both values the loss of polymerizability occurs faster than the release of bound ATP. Free ATP and free Ca^{2+} ions protect actin from inactivation at these pH values.

Previous studies from this laboratory [5, 6, 7] have shown that various proteins are able to bind ATP in acid medium. G-Actin is the only protein which contains bound ATP in neutral medium; its amounts correspond to one mole per mole of protein [30, 1, 13, 29]. During polymerization of G- to F-actin, bound ATP dephosphorylates to ADP and the latter remains bound to F-actin in the same proportions [23, 21, 30, 29]. The aim of the present study was to investigate how the changes of pH influence the binding of these nucleotides by actin and to what extent this protein possibly differs from other proteins.

In the previous experiments [5, 6] the amount of protein-bound ATP was determined after precipitation of proteins in the presence of ATP at various pH values either by adding ethanol or by heat denaturation. In the meantime a method has been developed [28, 1, 29] in which the free nucleotides are removed from actin solutions by Dowex 1 treatment. In the present work both procedures have been therefore applied to actin.

MATERIAL AND METHODS

Actin, free of tropomyosin, was extracted from acetone-dried muscle powder with water for 30 min. at 0° as previously described [8] and purified according to Mommaerts [20]. G-Actin, obtained by dialysis of F-actin pellets against 0.2 mM-ATP

in 2 mM-tris-HCl, pH 8.0, was used either native or heat-denatured for 10 min. at 100°. To the G-actin solutions containing various amounts of added ATP, acetate buffer for pH 3.6, 4.7 and 5.8 or tris-HCl for pH 7 and 8 was added and, after 10 min. incubation, the protein was precipitated by 2 vol. of 96% ethanol at 0°. After centrifugation the free nucleotides were washed out from the precipitated protein with the mixture of buffer solution used for incubation and 96% ethanol (1 : 2, v/v). Subsequently the bound nucleotides were liberated by treatment of the precipitate with perchloric acid. After centrifugation the amount of protein in the precipitate and the amount of the liberated nucleotide in the perchloric acid extracts were determined. Adenine nucleotides were determined either in a Unicam spectrophotometer SP 500 at 260 mμ or by ribose determination [18], and protein by biuret method according to Gornall *et al.* [12].

When the amount of bound nucleotide was determined with the use of Dowex 1, the G-actin solution with added ATP was adjusted to appropriate pH (in the acid pH range by 0.1 N-HCl at 0°) and, after 10 min. incubation, gently stirred for 3 min. with Dowex 1, chloride form (200-400 mesh) as previously described [9]. Before use the resin was washed successively with 1 N-NaOH, 1 N-HCl, with several portions of deionized water and finally with 2 mM-tris-HCl, pH 8.0.

Free bivalent cations were removed in a similar way by Dowex 50, tris form (200 - 400 mesh) [3], previously purified by successive washing with 1 N-HCl, 1 N-NaOH, water and finally with tris-HCl, pH 8.0.

Actin containing bound ^{45}Ca was prepared according to Bárány *et al.* [3] by incubation of G-actin (previously treated with Dowex 50) with $^{45}CaCl_2$ for 20 min. Radioactivity was measured using a Chicago Nuclear Corp. gas flow counter with a "Micromil" window.

Viscosity measurements were carried out in Oswald viscometers at 21°.

ATP (disodium salt) was obtained from Pabst Lab. (Milwaukee, U.S.A.) and $^{45}CaCl_2$ from the Radiochemical Centre, Amersham, England.

RESULTS

Table 1 shows the effect of pH on the amount of ATP (or ADP) bound by actin, either precipitated with ethanol or after Dowex 1 treatment. Under the conditions of experiments, at pH 5.8, 7.0 and 8.0 F-actin is formed from G-actin and ADP instead of ATP is bound by actin. This, however, does not influence the results in view of the lack of difference in the nucleotide binding between G- and F-forms, as mentioned in the introduction. The amount of the nucleotide found in the precipitated actin, both heat-denatured and untreated, markedly diminished as the pH increased from 3.6 to 4.7, i.e. to the isoelectric point of actin. When pH further increased, the ability of heat-denatured actin to bind nucleotides continued to decrease. Contrary to this, native actin at pH 5.8, 7.0 and 8.0 bound a much greater amount of nucleotide than at the isoelectric point. At these pH values the amount of bound nucleotide found in actin precipitated with ethanol (about 1 mole per mole of G-actin) corresponded to that obtained after Dowex 1 treatment. At pH

4.7 it was difficult to use Dowex for removal of free nucleotide because of precipitation of actin; at pH 3.6, however, contrary to the high increase of binding found by the precipitation method, the amount of ATP remaining bound after Dowex 1 treatment was small.

Table 1

The binding of ATP (or ADP resp.) by actin at various pH *values*

In experiments with precipitated actin, to 3-ml. samples containing actin solution (3.0 mg./ml.), 0.2 mM-ATP and 0.1 M buffer solution (acetate buffer in the pH range 3.6 - 5.8 and tris-HCl at pH 7.0 and 8.0) 2 vol. of 96% ethanol were added. The precipitated protein was separated by centrifugation, rinsed twice with a mixture of 0.1 M of the appropriate buffer and 96% ethanol (1 : 2, v/v), and finally treated with $HClO_4$. After centrifugation the amount of nucleotide in the supernatant and the amount of the protein in the precipitate were determined.

In the experiments with Dowex 1, samples of actin solution, previously adjusted to the required pH with 0.1 N-HCl in the presence of 0.2 mM-ATP, were treated with a suspension of resin. For details see Methods. The results are expressed as moles of bound nucleotide per 60 000 g. of actin; each value is the average of three parallel determinations.

pH	Nucleotide bound to actin		
	precipitated with ethanol		after stirring with Dowex 1
	untreated actin	heat-denatured actin	
3.6	1.81	1.90	0.45
4.7	0.48	0.40	—
5.8	0.97	0.14	1.18
7.0	1.05	0.12	1.07
8.0	0.99	0.09	—

In the experiments shown in Table 1 the concentration of free ATP present in actin solution was 0.2 mM. It was observed that when actin was precipitated in the presence of 1 mM-ATP the amount of bound ATP, especially at pH 3.6, was much greater. Therefore the effect of concentration of the added ATP on the amount of ATP bound by actin was examined. At pH 3.6 the amount of bound ATP considerably increased with the increase of ATP concentration (Fig. 1); at pH 4.7 this increase was much smaller. Presentation of the above data in form of relationship between the logarithms of bound and free ATP suggests that the character of binding resembles the adsorption isotherm (Fig. 1B).

Although in a neutral medium heat-denatured actin, like serum albumin, bound only very small amounts of nucleotides, the binding seemed to depend on the concentration of free ATP (Fig. 2), similarly as it was observed at lower pH values. Contrary to this, in the case of native F-actin the increase of the concentration of free ATP had no influence on the extent of binding. Thus, the binding of nucleotides by native F-actin at neutral pH showed a different character than at pH 3.6 and 4.7.

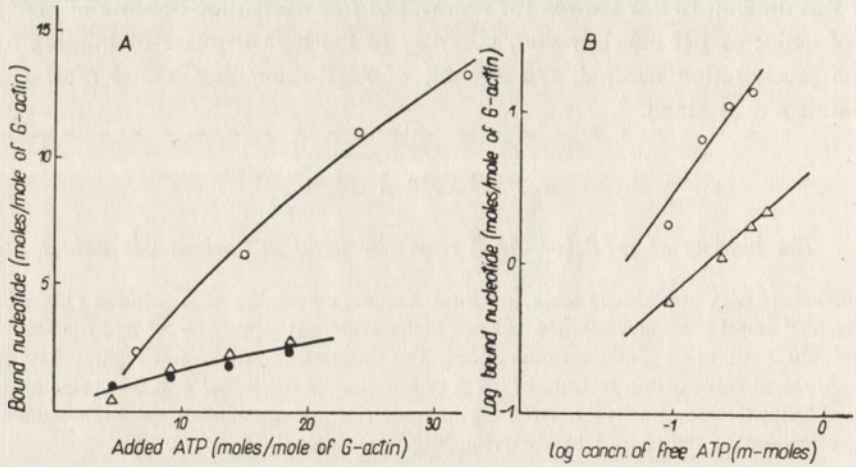

Fig. 1. The effect of ATP concentration on the binding of ATP by actin at pH 3.6 and 4.7. (*A*), To 3-ml. samples, containing actin solution (3.0 mg./ml.), ATP at the concentration indicated and 0.1 M-acetate buffer of the required pH, 2 vol. of ethanol was added, and the content of bound ATP was estimated. For details see Methods. (○), pH 3.6, untreated actin; (△), pH 4.7, untreated actin; (●), pH 4.7, heat-denatured actin. (*B*), The relationship between the logarithms of free and bound ATP. For calculation the data presented in Fig. 1A were used.

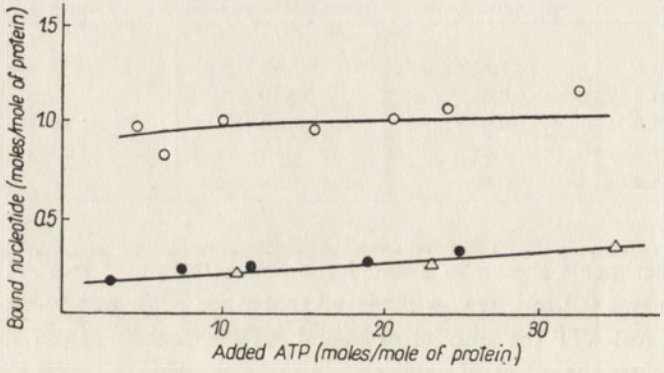

Fig. 2. The binding of nucleotide by actin and by serum albumin, in neutral medium. To 3-ml. samples, containing about 3.0 mg. of protein per ml. in 0.1 M-tris-HCl, pH 7.5, and ATP at the concentration indicated, 2 vol. of ethanol was added, and in the precipitate the content of bound nucleotide was determined. For details see Methods. (○), Native actin; (●), heat-denatured actin; (△), serum albumin.

It seemed interesting to examine how the binding at neutral pH was influenced by the exposure of actin to acidic or alkaline medium. The effect of such treatment on the ability to polymerize, another characteristic property of actin, was also examined. In these experiments the solution of G-actin, deprived of free ATP and bivalent cations by treatment with Dowex 1 and Dowex 50 respectively, was adjusted at 2° to pH 3.6 or 11.5. After various time intervals the samples were neutralized and the amount of the bound nucleotide after subsequent treatment with Dowex 1,

and viscosity after polymerization, were determined. Fig. 3 shows that both at pH 3.6 and 11.5 the loss of polymerizability occurred faster than the loss of ability to bind ATP. The addition of 0.2 mM-ATP decreased the rates of both processes;

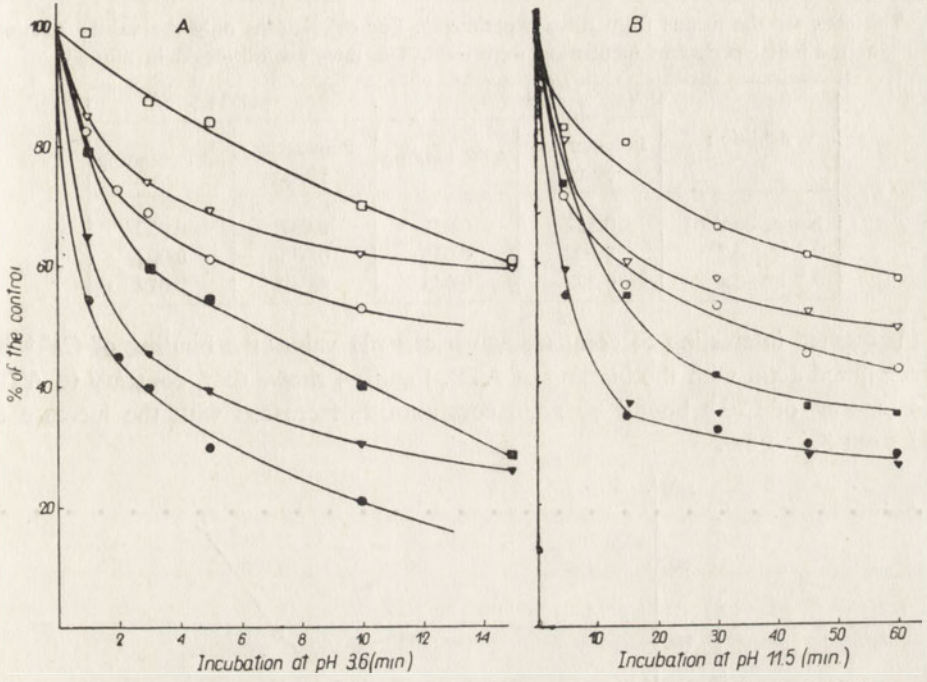

Fig. 3. The effect of incubation at (*A*), pH 3.6 and (*B*), pH 11.5, on the ability to bind ATP and on polymerizability of actin. To 10-ml. samples of actin (3.8 mg./ml.), previously treated with Dowex 1 and Dowex 50, either 0.1 N-HCl to pH 3.6 or 0.1 N-NaOH to pH 11.5 were added at 2°. (The amount of 0.1 N-HCl and 0.1 N-NaOH needed for adjusting the pH to 3.6 and 11.5, respectively, was determined on a pH-meter in a special control sample). After time intervals indicated, an equivalent amount of 0.1 N-NaOH (or HCl) and 10 mM-tris-HCl, pH 7.8, were added. Two 3-ml. portions of this solution were treated with Dowex 1 and, after removal of the resin, the content of bound ATP was determined as described in Methods. To the third 3-ml. sample 0.1 M-KCl and 1 mM-$MgCl_2$ were added and, after several hours, the viscosity was measured. The results for ATP binding and specific viscosity are expressed as percentage of the values for the control sample, i.e. the sample of original actin solution to which 0.05 M-KCl was added in amount equal to two volumes of the added 0.1 N-HCl or 0.1 N-NaOH. (○), (▽), (□), Nucleotide binding; (●), (▼), (■), polymerizability; (○, ●), no free ATP and Ca^{2+}; (▽, ▼), 0.2 mM-$CaCl_2$ added prior to HCl or NaOH; (□, ■), 0.2 mM-ATP added prior to HCl or NaOH.

0.2 mM free Ca^{2+} had somewhat smaller effect than ATP, whereas free Mg^{2+} was without effect (the results are not shown in the Figure). Both the loss of polymerizability and the release of bound ATP proceeded much faster at pH 3.6 (Fig. 3A) than at pH 11.5 (Fig. 3B). (Attention should be paid to the difference between the scales of abscissa in these Figures). Both processes seem to obey the first order kinetics at least at the initial period of incubation. Table 2 shows the approximate rates of the loss of polymerizability and of the release of bound ATP by actin during incubation at 2 - 3° at pH 3.6 and 11.5.

Table 2

Approximate rates of the loss of polymerizability and of the release of bound ATP by actin during incubation at pH 3.6 *and* 11.5 *in the presence of free ATP or free* Ca^{2+}

The data are the means from three experiments. For calculations only the values obtained at the initial period of incubation were used. The rates are expressed in $min.^{-1}$

Addition	pH 3.6		pH 11.5	
	Polymerizability	ATP binding	Polymerizability	ATP binding
None, control	0.532	0.058	0.087	0.020
0.2 mM-ATP	0.100	0.035	0.035	0.007
0.2 mM-Ca^{2+}	0.290	0.043	0.069	0.014

It seemed interesting to compare at various pH values the binding of Ca^{2+} by precipitated actin with the binding of ATP. Figure 4 shows that, contrary to ATP, the amount of Ca^{2+} bound by actin continuously increased with the increase of pH from 3.6 to 8.0.

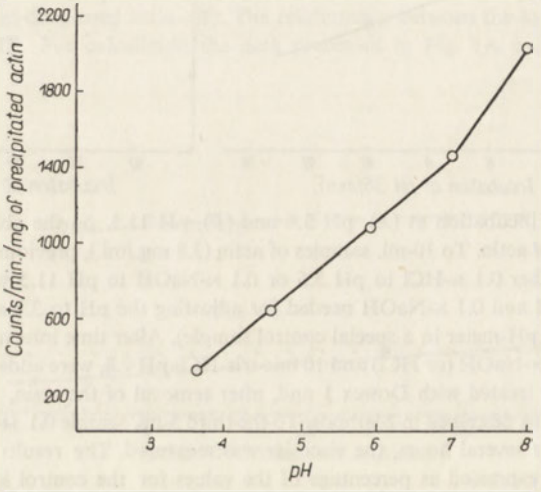

Fig. 4. The binding of ^{45}Ca by actin at various pH values. G-Actin solution, 3.9 mg./ml., was treated with Dowex 50 in the presence of 0.2 mM free ATP and, after removal of the resin, was incubated for 20 min. with 0.2 mM-$^{45}CaCl_2$. In 3-ml. samples the protein was precipitated with 2 vol. of ethanol in the presence of 0.1 M buffer of the required pH. The precipitates separated by centrifugation were rinsed with a mixture of 0.1 M of the appropriate buffer and 96% ethanol (1 : 2, v/v) and dissolved in 5 ml. of water containing one drop of conc. NaOH. Protein content and radioactivity were determined in the solution.

DISCUSSION

Previous studies [5, 7] showed that at the acid pH values similar amounts of nucleotides are bound by various proteins. It seems therefore reasonable to call this type of binding the "unspecific" binding. The present experiments indicate that

the binding of ATP by actin in acid medium, up to pH 4.7 (the isoelectric point), has the same character as binding by other proteins; the binding ability is not influenced by heat denaturation and markedly decreases with the increase of pH. Above the isoelectric point, however, only heat-denatured actin behaves like other proteins, i.e. the extent of binding diminishes further, whereas in the case of native actin an increase of binding at neutral pH is observed. In the latter case about 1 mole of bound nucleotide per 60 000 g. of actin was found in actin precipitated with ethanol. This value agrees well with the amount of bound nucleotide found with the use of the Dowex 1 method, both in the present work and in other papers [30, 1, 13, 29]. Since the binding of ATP (or ADP) around neutral pH is specific for actin only, it may be called the "specific" binding. Besides, at shese pH values the concentration of free ATP has practically no effect on the extent of binding. This observation is in good agreement with the studies of Mommaerts [21] and Ulbrecht *et al.* [30] who showed by ultracentrifugation of F-actin that the amount of bound ADP did not increase in the presence of higher concentrations of ATP.

The relation between the pH value and the ATP-binding ability of actin was investigated by Martonosi & Gouvea [15]. These authors, however, estimated the binding only at pH values about 3.6 and 7 to 8; by interpolation they obtained a straight-line decrease of binding with the increase of pH, and did not notice therefore the sharp minimum at the isoelectric point of actin, which has been observed in the present work. Several authors [23, 14, 26, 11] showed that, as a result of isoelectric precipitation, the nucleotides are removed from actin. Laki *et al.* [14] and Szent-Györgyi [26] applied the precipitation at pH 4.7 at 100° for quantitative liberation of the bound nucleotide. However, according to the results of the present work and in agreement with Ulbrecht *et al.* [30] the removal of nucleotide at the isoelectric point is not quantitative, even at 100°, at least after a single precipitation. The amount of nucleotide which remains bound depends on the initial concentration of ATP (cf. Fig. 1). Hence, when the purification of actin by isoelectric precipitation [23] is performed in the presence of ATP, fully active protein is obtained. On the other hand, the method of heat precipitation of F-actin at neutral pH, used by Asakura *et al.* [2] for separation of the nucleotide from protein, gives in the light of the presented results quantitative liberation of bound nucleotide from actin.

The ethanol precipitation method used in the present experiments can be applied only to F-actin since G-actin is not precipitated under those conditions. However, by ultrafiltration [22] it was possible to demonstrate that there was no difference in the binding between G- and F-actin at neutral pH [25]. It was found for serum albumin [7] that the unspecific binding of ATP could be observed not only in precipitated protein but also in protein in solution. Similarly, actin remaining in solution showed the same relationship between ATP binding and pH, as the precipitated actin.

In the experiments of Martonosi & Gouvea [15] after 30 min. incubation of actin at pH 3.0 or 11.5 no ATP binding was observed after subsequent neutralization. This phenomenon was accompanied by the loss of ability to polymerize. In the present work more details concerning these two processes have been obtained. Thus, it has

been found that the inactivation of actin proceeds much faster at acid than at alkaline pH. At both pH values the loss of polymerizability occurs faster than the release of bound ATP[1]. This phenomenons i somewhat similar to the effect of some mercurials on G-actin [9] . At both acid and alkaline pH values some rapid changes occur probably in the tertiary structure of actin. This view is in agreement with the recent observation of Mihashi & Ooi [19] who found that at pH about 11 an unfolding of actin molecule took place. The present results show that these changes lead first to the irreversible loss of polymerizability; the slower release of bound ATP seems to be rather the consequence of the former process.

Free ATP protects actin from the effects observed under the influence of acid or alkaline pH. It does not seem, however, that the effect of ATP can be simply explained by its unspecific binding to actin at acid pH. At pH 4.7 free ATP fully protects actin from inactivation — the phenomenon first observed by Straub & Feuer [23] — although at this pH the binding of ATP is rather small. On the other hand, at pH 3.6, when the protective effect of ATP is much weaker, actin binds considerable amounts of this nucleotide. Moreover, ATP protects actin from the inactivating effect of both hydrogen and hydroxyl ions, although the nucleotide is bound to actin only at acid pH values.

In general the effect of free ATP and free Ca^{2+} on one hand, and the lack of any effect of free Mg^{2+} on the other, is in agreement with many other observations on the protective effect of these compounds against the action of several agents causing inactivation of actin [17, 3, 16, 24, 10].

Contrary to the increased ATP binding at acid pH, as assayed by the precipitation method, the binding of calcium markedly diminishes when the pH is decreased below 7. The latter results, obtained with the method of precipitation of actin with ethanol, agree well with the previous observations of Bárány *et al.* [3] who studied the binding of Ca by centrifugation of actin.

On the other hand, the dependence of binding of Ca upon pH is similar to that of ATP binding, when the latter is assayed with the Dowex 1 method. This observation gives an additional proof of the close correlation between the specific binding of calcium and ATP by actin previously found by several authors under different conditions [15, 27, 3, 16, 24].

All previous results [5, 6, 7], viz. those connected with the effect of pH, addition of salts, various modifications of proteins, the influence of the number of phosphate groups in the nucleotide molecule etc., strongly support the view that in unspecific complexes of ATP and proteins an interaction between cationic groups of protein and anionic groups of nucleoside phosphates takes place. The fact that these complexes are decomposed by anionite Dowex 1 is also in agreement with this idea. The complex of actin with ATP formed at acid pH range seems to have the same unspecific character. The results of this work give further indication that the specific binding of

[1] The observations of Biro *et al.* [4] on the influence of incubation at pH 3 and 11 on the depolymerization of F-actin and on the release of bound ADP are in good agreement with the results of the present work, performed with G-actin.

nucleotides by native actin in a neutral medium exhibits a peculiar character. It is reasonable to assume that in the specific binding other functional groups of the actin molecule are involved. Our observations showing that the specific binding of ATP and the polymerization of actin are both much more sensitive towards hydrogen than towards hydroxyl ions provide some additional information leading to a better understanding of these two phenomena.

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ZALEŻNOŚĆ WIĄZANIA NUKLEOTYDÓW PRZEZ AKTYNĘ OD WARTOŚCI pH

Streszczenie

1. W zakresie pH od 3,6 do 4,7 kompleksy aktyny z ATP przypominają swym charakterem analogiczne kompleksy tworzone przez różne, uprzednio badane, białka; zdolność wiązania nukleotydów spada wraz ze wzrostem pH, ilość wiązanych nukleotydów wzrasta wraz ze wzrostem ich stężenia; denaturacja cieplna białka nie wpływa na zdolność wiązania ATP.

2. W przeciwieństwie do innych białek, rodzima F-aktyna wykazuje w środowisku obojętnym specyficzną zdolność wiązania nukleotydów adeninowych. Ilości nukleotydów wiązane w tych warunkach są większe niż w punkcie izoelektrycznym i praktycznie nie zależą od stężenia wolnych nukleotydów. Aktyna zdenaturowana termicznie traci prawie całkowicie zdolność wiązania nukleotydów w środowisku obojętnym.

3. Nieodwracalna inaktywacja aktyny zachodzi przy pH 3,6 szybciej niż przy pH 11,5. W obu przypadkach spadek zdolności do polimeryzacji jest szybszy niż utrata związanego ATP. Wolny ATP i wolny Ca^{2+} chronią aktynę przed inaktywacją przy obu podanych wyżej wartościach pH.

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UBIQUINONE IN PROLINE OXIDATION

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1. In rat liver and kidney mitochondria, endogenous ubiquinone is reduced in the presence of proline as substrate. The oxidation of proline is unaffected by arsenite and low concentration of amytal. The oxidation is not stimulated by exogenous NAD in fresh or NAD-depleted mitochondria. 2. In other rat tissues and in ox and pig liver and kidney mitochondria no oxidation of proline was observed. 3. The soluble preparation of proline dehydrogenase from rat liver mitochondria is able to reduce exogenous ubiquinone. 4. All the data indicate that proline dehydrogenase is a flavoprotein enzyme for which ubiquinone forms an essential link in the respiratory chain.

In 1935 Weil-Malherbe & Krebs [24] found that guinea pig liver and kidney oxidize proline to glutamic acid. Similar systems were found to be present in rabbit kidney [23] and rat liver [16, 8]. Lang & Lang [11] described a preparation obtained from rat liver, termed proline oxidase complex, which catalyses the oxidation of proline *via* NAD and the cytochrome system. More recently Johnson & Strecker [9] reported the existence of another system present in rat liver mitochondria which oxidizes L-proline stoicheiometrically to Δ^1-pyrroline-5-carboxylic acid. The system requires oxygen and cytochrome *c* but not nicotinamide adenine nucleotides. Since in this respect it is similar to succinate [8], α-glycerophosphate [20] and choline oxidases [3], it seemed interesting to see whether ubiquinone serves as an electron acceptor for the system described by Johnson & Strecker, as it does for other systems. A short report of this work has been presented [5].

MATERIAL AND METHODS

Wistar albino rats weighing 220 - 250 g. were used without prior starving. Mitochondria were prepared by differential centrifugation: liver mitochondria in a sucrose-EDTA medium as described by Szarkowska & Erecińska [21], kidney and brain mitochondria in sucrose-EDTA-TRA (triethanolaminehydrochloride) according to the method of Klingenberg *et al.* [10], skeletal muscle and heart mitochondria using crystalline proteinase as described by Bode & Klingenberg [1] and Szarkowska & Klingenberg [22]. NAD-depleted mitochondria were prepared according to the

procedure of Ernster & Navazio [6]. Phospholipid-depleted mitochondria were prepared using a mixture of acetone and water (9 : 1, v/v) as described by Lester & Fleischer [12].

Oxygen uptake and respiratory control were determined in 0.25 M-sucrose - 0.001 M-EDTA - 0.01 M-TRA, pH 7.4, at room temp. using a Clark oxygen electrode. The reduction of cytochrome *c* was measured at room temp. by the increase in extinction at 546 mμ in the recording Eppendorf photometer. The results were calculated using the millimolar extinction coefficient $9.8 \times cm.^{-1}$. Oxidized and reduced ubiquinone was determined as described previously [4] and exogenous ubiquinone as described by Szarkowska & Drabikowska [20].

Proline dehydrogenase was purified partially from the acetone-dried rat liver mitochondria. A 30% suspension of the acetone-dried powder in 0.06 M-phosphate buffer, pH 7.6, was homogenized in a glass-Teflon homogenizer, stirred for 15 min. at 0° and centrifuged for 30 min. at 18 000 *g*. The supernatant solution was decanted and discarded, and the extraction with phosphate buffer was repeated. Then the residue was homogenized with the same buffer containing 5 mM-proline. The vessel was gassed with N_2 for 5 min., and 1 mg. of *Bathrops atrox* venom (as a 0.5% solution in 0.06 M-phosphate buffer, pH 7.6) was added per 100 mg. of protein. The vessel was gassed with N_2 for additional 5 min., then tightly closed and the mixture incubated for 30 min. After incubation the suspension was cooled and centrifuged for 30 min. at 105 000 *g*. To the supernatant, solid ammonium sulphate was added to 0.4 saturation and after 30 min. centrifuged at 3000 *g* for 15 min. The precipitate was dissolved in 0.06 M-phosphate buffer, pH 7.6, and used for experiments.

Lecithin used for assays was prepared by Dr. T. Chojnacki from CMP-^{32}P-choline according to Chojnacki & Korzybski [2]. Inorganic phosphate was determined by the method of Strickland *et al.* [19].

Mitochondrial protein was determined by the biuret method as described by Szarkowska & Klingenberg [22] and calculated from the formula: 1 mg. protein = $= 17.5 \times E_{546m\mu} \times cm.^{-1}$ for the volume of 5 ml. [7]. In the soluble enzyme preparation the amount of protein was determined according to Lowry *et al.* [13].

All the chemicals used were commercial products: NAD, ADP and coenzyme Q_{10} of Sigma (St. Louis, Mo., U.S.A.), coenzyme Q_6 of Farmochimica Cutolo-Calosi S.p.a. (Napoli, Italy); proline, succinate, malate and glutamate were repacked in Gliwice (Poland). *Bathrops atrox* venom was kindly given to us by Professor Dr. D. Shugar, and Triton X-100 by Roem & Haas (Philadelphia, Pa., U.S.A.). Crystalline proteinase was a product of Novo Industrie A/S (West Germany).

RESULTS

Proline oxidation by rat liver and kidney mitochondria exhibited marked similarity; oxygen uptake in both tissues was quite efficient and respiratory control could be demonstrated (Table 1). Rat heart, brain and skeletal muscle mitochondria as well as ox liver and kidney and pig liver and kidney mitochondria did not oxidize

Table 1

Oxidation of proline by fresh mitochondria of rat liver and kidney

Oxygen uptake was measured in the incubation mixture containing 0.25 M-sucrose, 1 mM-EDTA, 10 mM-triethanolaminehydrochloride pH 7.4, 1 mM-P_i, 0.2 mM-ADP, 10 mM-proline and 5.3 mg. mitochondrial protein. For measurement of cytochrome *c* reduction, the incubation mixture contained 0.06 M-phosphate buffer, pH 7.4, 1 mM-KCN, 0.05 mM-cytochrome *c*, 10 mM-proline and 0.21 mg. mitochondrial protein. The results are expressed per 1 mg. protein per minute.

Mitochondria	μg. atom O_2	μmole cytochrome *c* reduced
Liver	0.044	0.036
Kidney	0.022	0.017

Table 2

Oxidation of different substrates by fresh and NAD-depleted rat kidney and liver mitochondria

Conditions as described in Table 1. Concentration of substrates, 10 mM; where indicated, 0.5 mM-NAD added. The results are expressed per 1 mg. protein per minute.

Additions	μg. atom O_2	μmole cytochrome *c* reduced	
	Kidney mitochondria		
	fresh	fresh	NAD-depleted
Succinate	0.109	0.158	0.331
Proline	0.022	0.016	0.050
Proline, NAD	0.022	0.016	0.050
Malate	0.011	0.009	0.006
Malate, NAD	0.019	0.021	0.094
Glutamate	0.015	0.009	0
Glutamate, NAD	0.025	0.021	0.061
	Liver mitochondria		
	fresh	fresh	NAD-depleted
Succinate	0.087	0.130	0.332
Proline	0.026	0.044	0.127
Proline, NAD	0.026	0.044	0.127
Malate	0.009	0.008	0.008
Malate, NAD	0.009	0.026	0.210
Glutamate	0.062	0.026	0
Glutamate, NAD	0.062	0.058	0.230

proline. Comparative data on the rates of oxidation of various substrates by fresh and NAD-depleted rat liver and kidney mitochondria are given in Table 2. It can be seen that the rate of proline oxidation was of the same order of magnitude as that of malate and glutamate. The addition of NAD to fresh or NAD-depleted

Table 3

Effect of inhibitors on the oxidation of different substrates by rat liver mitochondria

Conditions as described in Table 1.

Additions	μg. atom O_2/mg. protein/min.				
	Succinate	Proline		Malate	Glutamate
None	0.109	0.022	0.046*	0.011	0.045
1 mM-KCN	0	0	0	0	0
10 mM-Arsenite	0.109	0.022	0.046*	0	0
2 mM-Amytal	0.109	0.005	0.046*	0	0
0.4 mM-Amytal	0.109	0.020	0.046*	0	0

* NAD-depleted mitochondria.

Table 4

Reduction of endogenous ubiquinone in fresh and NAD-depleted rat kidney and liver mitochondria

Medium: sucrose - EDTA - triethanolaminehydrochloride, pH 7.4. Substrate concentration 10 mM; 1 mM-KCN and 5 mM-amytal added where indicated; mitochondria: kidney, fresh (10.2 mg. of protein), kidney, NAD-depleted (6.3 mg. of protein) or liver, NAD-depleted (7.2 mg. of protein). The incubation mixture was aerated for 7 min. prior to the addition of substrate, and the incubation carried out for 10 min. at 22°.

Additions	μmoles oxidized UQ/g. protein		
	Kidney mitochondria		Liver mitochondria
	fresh	NAD-depleted	NAD-depleted
None	1.68		
Amytal	1.68		
Amytal, KCN	1.64	3.03	2.90
Succinate	0.57		
Succinate, amytal	0.58		
Succinate, amytal, KCN	0.55		
Proline	1.68		
Proline, amytal	1.68		
Proline, amytal, KCN	0.83	1.35	0.83
Glutamate	1.47		
Glutamate, amytal	1.67		
Glutamate, amytal, KCN	1.90	3.00	2.76

mitochondria stimulated the oxidation of malate and glutamate, it was however without effect on proline oxidation.

In Table 3 are given the data concerning the effect of different inhibitors; 1 mM-KCN inhibited the oxidation of all the substrates tested, 10 mM-arsenite blocked

the oxidation of glutamate and malate and had no effect on succinate and proline, 2 mM-amytal depressed the rate of proline oxidation by about 80%, it had however no effect on this reaction in NAD-depleted mitochondria.

Proline reduced endogenous ubiquinone in the system inhibited by KCN (Table 4) but, contrary to succinate, it did not change the redox state of ubiquinone in freshly prepared mitochondria in the absence of KCN. Although amytal inhibited the oxygen uptake in the presence of proline by about 80%, it did not affect the reduction of endogenous ubiquinone by this substrate. Exogenous ubiquinone (UQ_6) could be reduced by proline in the presence of a soluble preparation of proline dehydrogenase (Table 5).

Table 5

Reduction of exogenous ubiquinone by soluble proline dehydrogenase

Medium: 10 mM-phosphate buffer, 1 mM-KCN, 10 mM-proline; Triton X100, 1.2 mg.; ubiquinone, 0.33 μmole; pH of the mixture 7.6, total volume 1 ml. Enzyme protein 0.580 mg.

Addition	μmole reduced UQ_6	Reduction (%)
None	0	0
Proline	0.242	76

Table 6

Effect of added ubiquinone on the rate of cytochrome c *reduction and oxygen uptake in lipid-depleted rat liver mitochondria*

Lipid-depleted mitochondria were prepared as described under Methods and suspended in sucrose--EDTA-triethanolaminehydrochloride medium. An appropriate amount of protein (7 mg. to measure O_2 uptake and 0.5 mg. for cytochrome *c* reduction) was suspended in 0.06 M-phosphate buffer and incubated for 10 min. at 30° in the presence of 0.05 mM-cytochrome *c*, 100 μg. of UQ_{10} and soluble mitochondrial lipids (4 μg. P/mg. protein). The control was incubated without UQ_{10}. Then proline was added, and KCN if the cytochrome *c* reduction was to be assayed. The results are expressed per 1 mg. protein per minute.

	μg. atom O_2	cytochrome *c* reduced
Without UQ_{10}	0.003	0.007
With UQ_{10}	0.011	0.025

The mitochondria depleted of phospholipids lose the capacity for electron transport. Lester & Fleischer [12] showed that the addition of ubiquinone and soluble mitochondrial phospholipids restores the capacity to oxidize succinate. Also in our experiments the addition of the same cofactors (Table 6) restored the oxidation of proline.

DISCUSSION

The presented data indicate that proline is oxidized mainly by rat liver and kidney mitochondria. This oxidation is unaffected by arsenite and 0.4 mM-amytal which inhibits completely the oxidation of all NAD-linked substrates. Amytal at higher concentration (2 mM) inhibits proline oxidation in fresh mitochondria probably because, as Roche & Ricaud [15] have shown, in fresh mitochondria about 80% of oxygen uptake with proline as substrate comes from Δ^1-pyrroline-5-carboxylic acid, which is a NAD-linked substrate. In NAD-depleted mitochondria the oxidation of proline is unaffected by 2 mM-amytal. Under such conditions proline is metabolized to Δ^1-pyrroline-5-carboxylic acid and this compound accumulates because it cannot be further oxidized in the absence of NAD.

The endogenous ubiquinone is reduced in the mitochondria in the presence of proline as substrate. The reduction takes place only in the presence of KCN and is not affected by amytal. This is additional evidence that amytal blocks the oxidation of Δ^1-pyrroline-5-carboxylic acid leaving the oxidation of proline untouched.

Proline dehydrogenase can be extracted from acetone-dried rat liver mitochondria after treatment with phospholipase. The procedure is similar to the isolation of another flavoprotein enzyme, choline dehydrogenase. The efficient oxidation of proline by NAD-depleted mitochondria, the lack of an inhibitory effect of arsenite and amytal, and the lack of a stimulatory effect of exogenous NAD, might indicate that both kidney and liver proline dehydrogenases are similar to the succinate [17], choline [14] and α-glycerophosphate [15] dehydrogenases of mitochondria. A number of flavoprotein enzymes are known for which ubiquinone forms an essential link in the respiratory chain. The reduction of endogenous ubiquinone by proline, the reduction of exogenous ubiquinone by the soluble enzyme and the restoration of the activity of phospholipid-depleted mitochondria by exogenous ubiquinone indicate that proline dehydrogenase belongs to this group of enzymes.

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UDZIAŁ UBICHINONU W UTLENIANIU PROLINY

Streszczenie

1. W mitochondriach nerki i wątroby szczura endogenny ubichinon ulega redukcji w obecności proliny. Utleniania proliny nie hamują arsenian ani amytal w niskim stężeniu. Egzogenny NAD nie stymuluje utleniania proliny ani w świeżych ani w pozbawionych NAD mitochondriach.

2. Mitochondria innych tkanek szczura oraz wątroby i nerki wołu i świni nie utleniają proliny.

3. Rozpuszczalny preparat dehydrogenazy proliny z wątroby szczura redukuje egzogenny ubichinon.

4. Dane wskazują, że dehydrogenaza proliny jest enzymem flawinowym, dla którego ubichinon jest niezbędnym ogniwem w łańcuchu oddechowym.

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DEPOLYMERIZATION OF RIBONUCLEIC ACID BY MITOCHONDRIAL BASIC PROTEIN OF HOG KIDNEY

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1. Mitochondrial basic protein from hog kidney displays depolymerase activity towards ribonucleic acid. 2. The effect of pH, temperature, substrate concentration and some ions on depolymerase activity has been studied.

Basic proteins extracted by dilute acids from nuclei or ribosomes, exhibit depolymerase activity towards ribonucleic acids [6]. In this laboratory, a basic protein containing about 30% of basic amino acids (mainly lysine) was isolated from hog kidney mitochondria by 0.2 M-sulphosalicylic acid extraction and CM-cellulose separation [11, 12]. In this paper, RNA depolymerase activity of the mitochondrial basic protein (MBP) is presented.

METHODS

Isolation of the mitochondrial basic protein from hog kidney was performed as described previously [11, 12].

For determination of RNA depolymerase activity, 1.5 mg. RNA was dissolved in 0.9 ml. of an appropriate 0.05 M-buffer, pH 6.9, and incubated at 37° with 0.1 ml. of aqueous solution of MBP (10 μg.); the blank sample contained 0.1 ml. of water. After 15 min. incubation, the undegraded RNA was precipitated with 3 ml. of a mixture of acetic acid - *tert.*-butanol (1 : 2, v/v) [3]. After 15 min. at room temperature the sediment was centrifuged off and in the supernatant the extinction at 260 mμ was determined against that of the blank sample.

RNA digestion products were separated by paper chromatography. For this purpose, to 1 ml. of 0.05 M-phosphate buffer, pH 6.9, containing 15 mg. RNA, was added 100 μg. of MBP or 3 μg. of pancreatic ribonuclease, and then water up to 10 ml. Then the incubation was carried out for 1 hr,. at 37°. Undegraded RNA was precipitated by adding 30 ml. of acetic acid - *tert.*-butanol mixture, centrifuged off and the supernatant dried at a temperature not exceeding 45°. The residue was dissolved in 0.2 ml. of water and 30 μl. of this solution was applied on a

30 cm. long strip of Whatman no. 1 paper and submitted to descending chromatography in 95% ethanol - 1 M-ammonium acetate (7 : 3, v/v) [9]. The RNA degradation products were located by photography in ultraviolet light.

Reagents: Yeast RNA (B.D.H., Poole, England) was purified according to Vischer & Chargaff [14] and dialysed before use for 24 hr. against 1 M-NaCl, then against water, and freeze-dried. Pancreatic ribonuclease was from Light, Colnbrook, England (chrom. purified, 50 KU/mg.) [5]. $MgSO_4$, $CaCl_2$, KCl, NaCl and other reagents were analytical grade.

RESULTS

The effect of varying conditions of incubation on depolymerization of RNA by MBP has been studied. The optimum pH (Fig. 1a) was at pH 6.7 - 7.0, therefore in further experiments the activity was assayed in 0.05 M-phosphate buffer at pH 6.9. The maximum velocity of the reaction (Fig. 1b) was obtained at RNA concentration not lower than 1.5 mg. per 1 ml. of the incubation mixture. In the presence of 1.5 mg. RNA per ml. the relation between the amount of the decomposed substrate and the amount of MBP was linear up to 20 μg. of MBP per ml. (Fig. 1c). Therefore in further experiments usually 10 μg. of MBP/ml. was applied. The relationship between the time of incubation and the amount of substrate decomposed was linear up to 15 min. (Fig. 1d); when longer time of incubation was applied, the increases in extinction became gradually smaller. The optimum tempera-

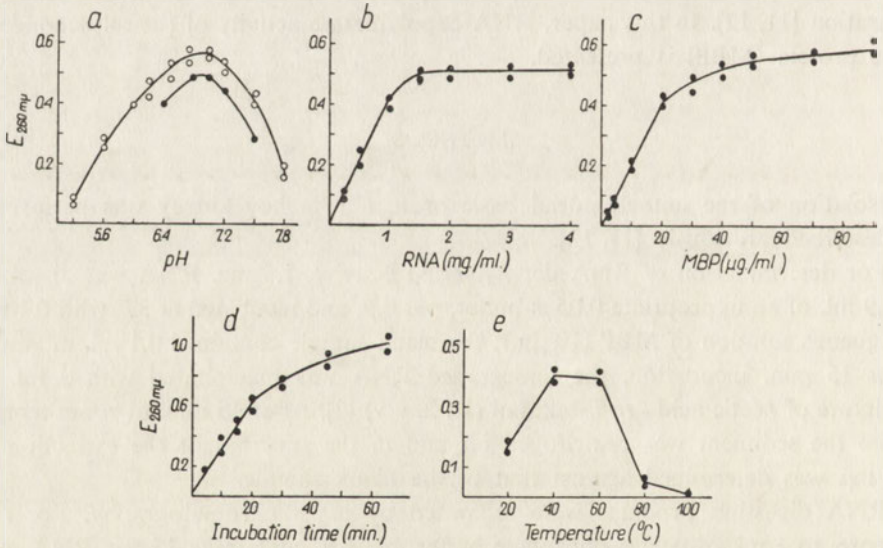

Fig. 1. Depolymerization of ribonucleic acid by mitochondrial basic protein (MBP) of hog kidney. Standard assay conditions: 1.5 mg. RNA was incubated with 10 μg. of MBP for 15 min. at pH 6.9 in 0.05 M-phosphate buffer, then the undegraded RNA was precipitated and in the supernatant the extinction was determined against that of a blank sample. *a*, Effect of pH: (●), in 0.05 M-phosphate buffer, (○), in 0.02 M-veronal - cacodylate buffer. *b*, Effect of RNA concentration. *c*, Effect of MBP concentration. *d*, Effect of time of incubation. *e*, Effect of temperature.

Table 1

The effect of ions on the depolymerization of ribonucleic acid by mitochondrial basic protein of hog kidney

Depolymerization of RNA was determined by measuring the extinction at 260 mμ after precipitation of the undegraded RNA in the mixture containing 1.5 mg. of RNA and 10 μg. of MBP incubated for 15 min. at 37° and pH 6.9. The effect of NaCl, KCl and phosphate was studied in 0.05 M-phosphate buffer, the effect of Ca^{2+} and Mg^{2+} in 0.05 M-arsenate buffer. The figures represent values of extinction at 260 mμ.

Salt added	Salt concn. (M)				
	0	0.005	0.05	0.1	0.2
NaCl	0.45	—	0.45	0.40	0.30
KCl	0.45	—	0.45	0.42	0.32
$CaCl_2$	0.53	0.38	0.18	0.11	—
$MgSO_4$	0.60	0.50	0.13	0.06	—
PO_4	0.45	—	0.45	0.42	0.22

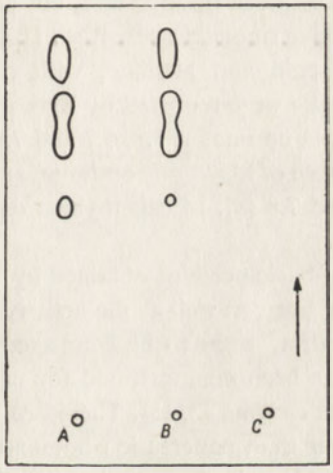

Fig. 2. Diagram of the photogram in ultraviolet light of the chromatogram of RNA degradation products after treatment with (*A*) mitochondrial basic protein from hog kidney and (*B*), pancreatic ribonuclease; (*C*), blank sample. For details see text.

ture varied between 40 and 60° (Fig. 2e); at 20° the activity amounted to 20% of the activity at 40°, at 80° it amounted to 5% of the optimum, whereas at 100° no activity was observed.

When RNA depolymerase activity was assayed at 37° in a MBP solution that had been heated at 100° for 60 min., only a 50% decrease in activity was found; this indicates that MBP is a relatively thermostable protein.

The effect of some anions and cations is shown in Table 1. Na^+, K^+ and Cl^- ions affected the activity but slightly, whereas Ca^{2+} and Mg^{2+} ions inhibited it to a large extent; at 0.1 M concentration 80% and 90% inhibition, respectively, was

observed. The phosphate ion had a similar effect at higher concentration (0.2M), the activity being inhibited by about 50%.

Figure 2 presents a diagram of the chromatogram of RNA degradation products after digestion with MBP or pancreatic ribonuclease. In the control sample (incubated without protein) no ultraviolet-absorbing spots were found, whereas in the proper samples acid-soluble products were present. On chromatography in the applied solvent system the products obtained by treatment either with MBP or pancreatic ribonuclease moved with similar velocity; it is not certain, however, that in either case the same products were formed.

DISCUSSION

Mitochondrial basic protein extracted with 0.2 M-sulphosalicylic acid from hog kidney, possesses RNA deplomerase activity. Paper chromatography of the RNA degradation products obtained after treatment with MBP or pancreatic ribonuclease permits to suggest that in either case both mononucleotides and oligonucleotides are formed. The pH optimum for the activity of MBP is similar to that reported by Kaplan & Heppel [4] for calf spleen ribonuclease, but different from the pH optima reported for other intracellular ribonucleases. Roth [10] has found in rat liver two intracellular ribonucleases, acid and alkaline, with pH optima, respectively, of pH 5.8 and 7.8. Similar results were reported by Zytko *et al.* [15]. Maver & Greco, using ox liver, found three pH optima, at 5.8, 6.7 and 7.8; however, when the assays were carried out in the presence of Mg^{2+} one optimum at pH 7.3 was obtained instead of the optima at pH 6.7 and 7.8 [8]. In calf thymus only one optimum was found at pH about 4.5-5.0 [7, 1].

Depolymerase activity of basic proteins obtained by Martin *et al.* [6] from nuclei and ribosomes of guinea pig liver, as well as the activity of the studied basic protein from hog kidney mitochondria, seem to be connected with the presence of basic amino acids which, as it has been demonstrated for pancreatic ribonuclease, form part of the active site of the enzyme [2, 13]. Therefore it seems possible to suggest that also some proteins other than pancreatic ribonuclease but possessing a similar sequence of basic amino acid residues, may have a similar activity.

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DEPOLIMERYZACJA KWASU RYBONUKLEINOWEGO PRZEZ ZASADOWE BIAŁKO MITOCHONDRIALNE NERKI WIEPRZA

Streszczenie

1. Zasadowe białko mitochondrialne nerki wieprza wykazuje własności depolimeryzacyjne wobec kwasu rybonukleinowego.

2. Przebadano wpływ pH, temperatury, stężenia substratu oraz niektórych jonów na aktywność depolimerazy.

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THE EFFECT OF BASIC PROTEINS ON THE OXIDATION OF EXOGENOUS $NADH_2$ IN RAT LIVER MITOCHONDRIA

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1. Protamine and mitochondrial basic protein (MBP) inhibited the oxidation of $NADH_2$ by mitochondria which were damaged by being suspended in hypo-osmotic solutions. 2. In intact mitochondria the oxidation of $NADH_2$ was stimulated by MBP and inhibited by protamine. In the presence of vitamin K_3 the inhibitory effect of protamine was not observed.

Basic proteins bind with mitochondria [3, 4] and are able to affect a number of processes occurring in mitochondria, such as oxidative phosphorylations, swelling and electron transport. This last process, which has been known to be inhibited by basic proteins [6, 7, 8], was recently found by Schwartz [10] to be stimulated in intact mitochondria by low concentrations of basic proteins.

In the present work, the effect of two basic proteins, namely protamine and hog liver mitochondrial basic protein [9], on the oxidation of exogenous $NADH_2$ in rat liver mitochondria was studied in relation to the degree of damage to the mitochondrial structure. To observe the possible differences in the mechanism of action of the basic proteins in undamaged and damaged mitochondria, vitamin K_3 (menadion) was added to open the pathway through the mitochondrial DT diaphorase [2, 1].

MATERIAL AND METHODS

The liver of a Wistar rat was homogenized manually in a Potter homogenizer and the mitochondria were isolated in 0.25 M-sucrose according to Weinbach [11]. The mitochondria isolated from one liver were suspended in 3 ml. of 0.25 M-sucrose and divided into three portions; each of them was submitted to a different treatment so as to obtain preparations with increasingly damaged mitochondrial structure. (A), Relatively undamaged mitochondria: 1.5 ml. of the suspension was incubated for 30 - 45 min. at room temp. and then used for experiments. (B), Moderately damaged mitochondria: to 1 ml. of the suspension 4 ml. of 0.125 M-sucrose was added and the

mixture used for experiments. (C), Highly damaged mitochondria: to 0.5 ml. of the suspension 4.5 ml. of water was added and the mixture incubated for 15 min. in a water bath at 30°.

The evaluation of the degree of damage to the mitochondrial structure was based on the extinction at 520 mμ per 1 mg. of mitochondrial protein. The measurements were made in 1 cm. light-path cells in a medium consisting of 1 mM-Na-phosphate buffer, pH 7.4, 0.5 mM-EDTA, pH 7.4, and 0.25 M-sucrose.

The activity of $NADH_2$ dehydrogenase was determined at 30° by measuring the decrease in extinction at 340 mμ with time, against a blank sample containing no $NADH_2$. The incubation medium (3 ml.) was composed of 1 mM-Na-phosphate buffer, pH 7.4, 0.5 mM-EDTA, pH 7.4, and 0.25 M-sucrose. Mitochondrial preparation A was added in amounts corresponding to 2 - 4 mg. of the mitochondrial protein, preparation B in 5-fold smaller, and C in 10-fold smaller amounts. Mitochondrial basic protein (MBP) extracted with sulphosalicylic acid from hog kidney [9] and protamine were added in relation to the amount of the mitochondrial protein. It was, depending on the mitochondrial preparation used, 75 - 150 μg. of the basic protein per 1 mg. of the mitochondrial protein. The solutions of basic proteins were adjusted prior to use to pH 7.4. Vitamin K_3 was applied at a concentration of 20 μM. The reaction was started by adding 0.3 μmole of $NADH_2$, and the determinations continued for 2 - 10 min. The values of the decrease in extinction were calculated per 1 mg. of the mitochondrial protein and per 10 min.

The content of protein in the mitochondria was determined by the biuret method, 1% natrium deoxycholate being added to obtain clear solutions [5].

Reagents used. Protamine sulphate (B.D.H., Poole, England), $NADH_2$ (Sigma, St. Louis, USA), EDTA and sucrose (Biuro Obrotu Odczynnikami, Gliwice, Poland), *prim.*- and *sec.*-sodium phosphate (Xenon, Łódź, Poland), vitamin K_3 (Tarchomińskie Zakłady Farmaceutyczne, Warszawa, Poland). Aqueous solution of sucrose was deionized on Amberlite IRC 50. Twice distilled and Amberlite IRC 50 deionized water was used throughout.

RESULTS

The addition of vitamin K_3 to the mitochondria mediates the electron pathway for the NAD-dependent substrates through mitochondrial DT diaphorase and to the vicinity of cytochrome *b* [1]. In the present experiments, the effect of vitamin K_3 on the oxidation of exogenous $NADH_2$ in mitochondrial preparations was dependent on the degree of damage to the mitochondrial structure (Table 1). In preparation A, in which the mitochondria were almost intact, vitamin K_3 had a marked stimulating effect; this effect was smaller in the more damaged mitochondria of preparation B and was absent in preparation C.

The effect of protamine alone and protamine in the presence of vitamin K_3 is shown in Table 2. It may be seen that protamine inhibited the oxidation of $NADH_2$ in all three mitochondrial preparations. In the undamaged preparation A the inhibition caused by protamine did not appear when protamine was added simultaneously

Table 1

The effect of vitamin K_3 on the oxidation of exogenous $NADH_2$ by undamaged and damaged rat liver mitochondria

The activity of $NADH_2$ oxidase was assayed by measuring the decrease in extinction at 340 mμ in a medium consisting of 1 mM-phosphate buffer, pH 7.4, 0.5 mM-EDTA, pH 7.4 and 0.25 M-sucrose. Concn. of vitamin K_3, 20 μM. The results are expressed as decrease in extinction/10 min./1 mg. of mitochondrial protein. The damage of the mitochondrial structure is expressed by the values of extinction at 520 mμ calculated per 1 mg. protein.

Mitochondria	Degree of damage (E_{520}/mg. protein)	Expt. no.	Activity of $NADH_2$ dehydrogenase ($-\Delta E_{340}$/10 min./mg. protein)	
			Control	Vit. K_3 added
A, Undamaged	0.55 - 0.45	1	0.06	0.25
		2	0.05	0.24
		3	0.06	0.18
B, Moderately damaged	0.30 - 0.20	1	0.52	0.93
		2	0.56	0.81
		3	0.55	0.80
C, Highly damaged	0.10 - 0.05	1	3.6	3.6
		2	2.4	2.8
		3	2.9	2.7

Table 2

The effect of protamine and vitamin K_3 on the oxidation of exogenous $NADH_2$ by undamaged and damaged rat liver mitochondria

The conditions of experiment as in Table 1. Protamine was added in amounts corresponding to those of the mitochondrial protein, 300, 60 and 30 μg. per sample for preparations A, B and C, resp. Concn. of vitamin K_3, 20 μM.

Mitochondria	Degree of damage (E_{520}/mg. protein)	Expt. no.	Activity of $NADH_2$ dehydrogenase ($-\Delta E_{340}$/10 min./mg. protein)		
			Control	Protamine added	Protamine and vit. K_3 added
A, Undamaged	0.55 - 0.45	1	0.06	0.02	0.08
		2	0.05	0.02	0.11
		3	0.06	0.03	0.15
B, Moderately damaged	0.30 - 0.20	1	0.56	0.08	0.16
		2	0.52	0.10	0.22
		3	0.55	0.10	0.30
C, Highly damaged	0.10 - 0.05	1	3.6	0.08	0.08
		2	2.4	0.12	0.20
		3	2.9	0.08	0.08

Table 3

The effect of mitochondrial basic protein (MBP) on the oxidation of exogenous $NADH_2$ by undamaged and damaged rat liver mitochondria

The conditions of experiments as in Table 1. MBP was added in amounts corresponding to those of the mitochondrial protein, 300, 60 and 30 μg. per sample for preparations A, B and C, resp.

Mitochondria	Degree of damage (E_{520}/mg. protein)	Expt. no.	Activity of $NADH_2$ dehydrogenase ($-\Delta E_{340}$/10 min./mg. protein)	
			Control	MBP added
A, Undamaged	0.55 - 0.45	1	0.06	0.24
		2	0.06	0.20
		3	0.05	0.34
		4	0.05	0.22
B, Moderately damaged	0.30 - 0.20	1	0.56	0.20
		2	0.52	0.20
		3	0.55	0.24
C, Highly damaged	0.10 - 0.05	1	3.6	1.4
		2	3.6	1.5
		3	2.4	1.0

with vitamin K_3. In the damaged mitochondria, vitamin K_3 had a small effect (preparation B) or had no effect at all (preparation C).

MBP applied in the same concentrations as protamine inhibited the oxidation of $NADH_2$ only in the damaged mitochondria (Table 3), whereas it had a distinctly stimulating effect on intact mitochondria.

DISCUSSION

The oxidation of exogenous $NADH_2$ by mitochondria is limited by permeability of the mitochondrial membrane. In undamaged mitochondria vitamin K_3 stimulated the oxidation by mediating the electron transport to the respiratory chain. Damage to the mitochondrial structure abolished the stimulating effect of the mediator (Table 1) by facilitating the contact of the substrate with the interior of the mitochondrion. The increase in the oxidation of $NADH_2$ after addition of vitamin K_3 to undamaged mitochondria despite the presence of protamine in the medium (Table 2), suggests that there is no blocking of the mitochondrial enzymic systems and that protamine reacts probably with the negatively charged components of the mitochondrial membrane, causing a decrease of its permeability for the substrate. In the damaged mitochondria, the inhibitory effect of basic proteins is not abolished by vitamin K_3. This suggests that in this case basic proteins become bound to the acidic components of the respiratory chain.

Whereas the binding of the basic proteins to intramitochondrial acidic components, independently of the kind and concentration of the basic protein, always

leads to inhibition of the oxido-reduction reactions, the binding of basic proteins to the mitochondrial membrane not always results in the inhibition of respiration. This is in agreement with the results showing that MBP stimulated the oxidation of exogenous $NADH_2$ by undamaged mitochondria (Table 3) and with similar observations of Schwartz [10] concerning the stimulation of respiration by low concentrations of histones.

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WPŁYW BIAŁEK ZASADOWYCH NA UTLENIANIE EGZOGENNEGO $NADH_2$ W MITOCHONDRIACH WĄTROBY SZCZURA

Streszczenie

1. Protamina i mitochondrialne białko zasadowe (MBP) hamowały utlenianie $NADH_2$ w mitochondriach uszkodzonych przez zawieszenie w roztworach hipoosmotycznych.

2. W nieuszkodzonych mitochondriach utlenianie $NADH_2$ było stymulowane przez MBP i hamowane przez protaminę. W obecności witaminy K_3 nie obserwowano hamowania przez protaminę.

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CHANGES IN THE CONTENT OF FREE AMINO ACIDS IN PIG BLOOD PLASMA AFTER INGESTION OF FOOD

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1. After ingestion of a half-synthetic diet supplemented with different proteins, a decrease in the concentration of individual amino acids in pig plasma followed by an increase was observed. 2. After feeding protein of high biological value, both the decreases and the maxima were very marked and appeared almost at the same time for all amino acids. Protein of lower biological value gave less distinct decreases and maxima, which moreover appeared at various time intervals. 3. After addition of L-lysine to the diet, a strong lysine maximum appeared earlier than the maxima for other amino acids.

The nutritive value of food protein depends not only on its amino acid composition but also on the availability of the amino acids. Evaluation of the absorption of essential amino acids is the most reliable criterion of the biological value of protein. Denton & Elvehjem [2] studied the changes in the concentration of essential amino acids in dog blood plasma after feeding zein, casein or beef meat. Longenecker & Hause [6] carried out similar experiments on dogs after feeding wheat gluten or gelatin with or without the addition of, respectively, L-lysine or L-tryptophan; and on dog and man [7] using wheat gluten. Recently Smith & Scott [11, 12] estimated the content of free amino acids in pooled samples of blood plasma obtained from groups of a few day old chickens. In this way they were able to study several kinds of protein on a considerable number of individuals using, however, only small amounts of blood.

In such experiments the time interval between the ingestion of the test meal and withdrawing of blood is of great importance. The most suitable would be the time when the plasma concentration of all, or at least a majority of amino acids is at a maximum. The results of Longenecker & Hause [6] showed that the maxima for the individual amino acids do not appear at the same time, but after an interval varying from 1 to 5 hr. In later experiments Longenecker & Hause [7] obtained somewhat differing results; in man the highest content of a majority of amino acids appeared at 2 hr., and in the dog at 3 hr. Denton & Elvehjem [2] observed that the maxima tend to appear earlier in the plasma of blood taken from the portal vein

than taken from the radial vein. They observed also a decrease in the concentrations of amino acids at 1 and 2.5 hr. after ingestion of zein.

Little information is available in the literature on the changes in the content of free amino acids in pig blood plasma after ingestion of food. Chance *et al.* [1] reported briefly the results of amino acid determinations in pig plasma. The paper of Long *et al.* [5], which appeared after the presented experiments had been completed, reports on the content of free amino acids in pig plasma within a few hours after ingestion of a meal supplemented with amino acids. Richardson *et al.* [10] studied the content of amino acids in relation to the time of starvation and the kind and amount of protein in the diet.

In the present work the changes in the content of amino acids in deproteinized pig blood plasma at various time intervals after feeding a half-synthetic diet supplemented with several kinds of protein, were studied.

MATERIAL AND METHODS

The experiments were carried out on three pigs of the variety Wielka biała (Great white): no. 62 (female, 145 kg.), no. 63 (male, 140 kg.) and no. 64 (female, 135 kg.). The pigs were starved for 18 hr., then fed a ration of 1.6 kg. containing the protein studied; this was eaten up within 10 - 15 min. The blood was withdrawn just before feeding (zero time) and then at 1, 2, 3 and 5 hr. after feeding. The following proteins were applied: fish meal with and without the addition of L-lysine (22.4 g. of monohydrochloride of L-lysine, product of Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan); overheated fish meal (2 hr. at 120°) with and without the addition of L-lysine; blood meal; and cracked corn. The protein amounted to 10% of the diet; the rest was a mixture consisting of: cellulose, 5%; wheat starch, 49%; vitamin mixture, 0.36%; mineral mixture, 1.32%; crude oxyterracine, 0.05%; calcium carbonate, 0.63%; linseed oil, 3.5%; cod liver oil, 0.71%; glucose, 39.43%. The mineral and vitamin mixtures contained, respectively: potassium chloride, 22.05%; salt, 38.32%; magnesium sulphate, 19.4%; ferric sulphate, 0.75%; zink sulphate, 0.8%; manganese sulphate, 0.53%; cobalt sulphate, 0.07%; copper sulphate, 0.05%; potassium iodide, 0.04%; calcium carbonate, 17.99%; vitamin A (325 I.U./1 g.), 2.64%; vitamin D (80 000 I.U./1 g.) 1.43%; vitamin E (tocopherol, 25%), 14.33%; vitamin B_{12} (0.1%), 3.25%; vitamin K_3 (menadion), 0.72%; vitamin B_6, 0.29%; vitamin B_2, 0.43%; choline chloride, 71.61%; nicotinic acid, 2.86%; calcium pantothenate, 2.15%; and thiamine, 0.29%.

About 50 ml. of blood withdrawn from the tail of the animal directly to a heparinized 100-ml. tube, was centrifuged for 15 min. at 3000 rev./min., the plasma carefully removed and centrifuged again for 25 min. at 4500 rev./min. Ten ml. of the plasma was treated with 1% picric acid, the precipitated protein centrifuged off, and the excess of picric acid removed according to Stein & Moore [13]. The sample was dried in a vacuum desiccator over sodium hydroxide, then dissolved in 5 ml. of citrate buffer, pH 2.2, and the amino acid composition was determined by ion-exchange column chromatography.

For separation of amino acids, the improved method of Moore, Spackman & Stein published in 1958 [8] was applied, with the modification of Kominz [4] consisting in using for the separation of basic amino acids a column 50 cm. in height instead of 15 cm. To higher columns 2 ml. of the tested solution and to lower ones 1 ml. was applied. For determination of amino acids in the effluent, the ninhydrin reagent in the modification of Moore & Stein [9] was employed.

More than thirty peaks were obtained but only fifteen amino acids listed in the Tables were quantitatively estimated. Glycine and alanine were determined together because their concentrations were so high that in a number of determinations their separation did not occur. For calculations, the average extinction coefficient [9] and average molecular weight of these two amino acids were taken.

RESULTS AND DISCUSSION

The values of blood plasma amino acids in three pigs after 18-hr. starvation, are summarized in Table 1. After ingestion of a test meal, two phenomena were observed: a decrease in relation to the starving value, and then an increase in the content of each amino acid studied, which were both dependent on the kind of protein in the diet.

Table 1

The content of free amino acids in pig blood plasma after 18-hr. starvation

Average values from 6 determinations on 3 animals are given; in parentheses the limit values.

Amino acid	mg./100 ml. of plasma	Amino acid	mg./100 ml. of plasma
Aspartic acid	0.5 (0.4 - 0.7)	Methionine	0.5 (0.2 - 0.9)
Threonine	1.5 (0.8 - 2.3)	Isoleucine	2.2 (1.5 - 3.2)
Serine	2.1 (1.6 - 3.2)	Leucine	2.4 (2.2 - 2.9)
Glutamic acid	2.9 (1.9 - 4.6)	Tyrosine	1.1 (0.5 - 2.2)
Proline	1.9*(1.6 - 2.4)	Phenylalanine	1.1 (0.9 - 1.6)
Glycine+alanine	9.8 (7.3 - 11.8)	Lysine	6.0 (3.5 - 7.9)
Valine	3.8 (3.2 - 4.6)	Histidine	2.3 (1.6 - 3.5)

* Average from 4 determinations.

After ingestion of protein of high biological value, such as fish meal (Table 2), the content of individual amino acids was distinctly lower and this decrease appeared at the first hour after feeding. The maximum amounts were observed for a majority of amino acids at 3 hr. For the rest of amino acids, the highest values appeared at 2 or 5 hr., the differences between the maxima and the values at 3 hr. being relatively small.

On addition of lysine to the fish meal, the decreases became less distinct and they appeared after 1 and 2 hr., or even, for glutamic acid, after 3 hr., whereas the maxima at 2 and 5 hr. were observed more often than previously. A strongly marked ma-

Table 2

Changes in the content of amino acids in pig blood plasma after feeding fish meal, and the effect o lysine

The values are expressed as mg./100 ml. of plasma.

Amino acid	Pig. no. 63, male					Pig. no. 62, female; lysine added				
	Time after feeding (hr.)									
	0	1	2	3	5	0	1	2	3	5
Aspartic acid	0.5	0.5	0.4	0.7	0.1	0.5	0.6	0.7	0.5	1.0
Threonine	2.3	2.1	2.4	3.0	3.2	1.4	1.1	1.3	1.5	1.9
Serine	3.2	1.6	2.3	2.8	2.6	1.9	1.4	2.0	2.4	1.8
Glutamic acid	1.9	1.8	2.4	2.0	2.1	4.6	4.3	3.2	2.6	4.7
Proline	1.6	—	2.3	3.2	2.9	2.4	1.4	3.3	3.0	3.0
Glycine+alanine	11.8	8.5	10.1	10.4	9.7	9.8	8.1	8.0	10.1	9.3
Valine	4.6	3.0	4.3	4.4	3.8	3.2	2.5	3.7	3.9	3.7
Methionine	0.9	0.2	0.4	0.9	0.8	0.4	0.1	0.4	0.3	0.4
Isoleucine	2.4	1.2	1.7	2.2	2.0	3.2	2.3	2.5	2.7	2.4
Leucine	2.6	1.6	2.5	3.2	2.7	2.9	1.9	2.1	2.3	2.6
Tyrosine	2.2	trace	1.4	1.7	1.9	1.0	1.2	1.4	1.1	1.0
Phenylalanine	1.0	trace	1.2	1.3	1.2	1.6	1.4	0.8	1.0	1.0
Lysine	7.9	1.0	1.0	7.7	6.7	5.3	3.0	7.4	4.6	7.2
Histidine	3.5	2.3	2.3	—	2.3	1.6	0.9	1.1	2.5	2.5

Table 3

Changes in the content of amino acids in pig blood plasma after feeding overheated fish meal, and the effect of lysine

The values are expressed as mg./100 ml. of plasma.

Amino acid	Pig. no. 64, female					Pig no. 63, male; lysine added				
	Time after feeding (hr.)									
	0	1	2	3	5	0	1	2	3	5
Aspartic acid	0.7	0.1	0.5	0.7	0.5	0.5	1.1	0.5	0.7	0.2
Threonine	0.8	1.3	1.0	1.2	1.1	1.8	2.5	3.1	2.8	3.3
Serine	1.6	2.7	2.3	2.0	2.2	2.3	1.5	2.1	2.5	2.4
Glutamic acid	2.9	4.2	2.8	3.0	3.0	2.5	3.0	3.0	3.6	2.3
Proline	1.9	2.3	2.8	1.6	2.1	—	2.9	—	2.5	4.3
Glycine+alanine	9.0	9.3	8.6	7.8	7.0	11.6	14.5	15.7	14.3	14.4
Valine	3.3	3.9	2.9	3.5	3.6	4.3	3.8	3.8	4.3	4.3
Methionine	0.2	0.1	0.2	0.4	0.4	0.8	0.7	0.7	0.7	0.7
Isoleucine	1.5	1.6	1.9	1.8	2.0	2.1	1.9	2.0	2.4	2.5
Leucine	2.2	2.1	2.2	2.4	1.5	2.4	2.0	2.2	2.4	3.2
Tyrosine	0.5	0.8	1.0	0.7	0.8	1.4	1.1	1.3	1.2	2.1
Phenylalanine	0.9	0.8	1.1	1.0	0.8	1.0	0.8	1.3	0.9	1.6
Lysine	5.1	3.7	3.2	3.9	2.7	7.8	4.4	8.4	6.7	6.9
Histidine	1.6	1.4	1.7	2.2	1.8	2.6	4.0	1.1	1.5	1.5

ximum for lysine appeared at 2 hr. For all amino acids, excepting glutamic acid and lysine, the differences between the maxima and the values at 3 hr. were not very great.

The overheating of the fish meal added to the diet affected the concentration patterns of practically all the amino acids; the changes were apparent especially after the addition of lysine (Table 3), the maximum value for lysine being the only one which appeared at the same time (2 hr.) as in the previous experiment.

After feeding cracked corn (Table 4) which contains protein of lower biological value but not subjected to harmful treatment, the amino acid concentration curves were somewhat similar to those observed after feeding raw fish meal. The decreases for almost all amino acids were marked and appeared at 1 hr. The maxima, however, were less distinct and appeared at 2 and 5 hr., their values being close to those for 3 hr.

Table 4

Changes in the content of amino acids in pig blood plasma after feeding cracked corn or blood meal

The values are expressed as mg./100 ml. of plasma

Amino acid	Pig no. 62, female; cracked corn					Pig. no. 63, male; blood meal				
	Time after feeding (hr.)									
	0	1	2	3	5	0	1	2	3	5
Aspartic acid	0.4	0.8	0.5	0.9	0.5	0.5	0.6	0.3	0.7	0.5
Threonine	1.2	1.0	1.5	1.0	1.0	1.6	1.4	0.9	1.3	1.5
Serine	1.9	1.7	1.9	1.7	1.6	1.8	1.5	1.1	1.6	1.3
Glutamic acid	3.4	2.4	3.7	2.9	2.4	2.2	2.0	1.2	2.3	2.2
Proline	—	2.0	2.7	2.9	3.4	1.7	—	2.1	2.8	3.1
Glycine+alanine	9.5	9.5	9.7	9.1	8.0	7.3	5.7	4.0	7.6	6.3
Valine	3.3	2.5	3.2	2.8	3.2	4.0	3.0	2.9	3.3	3.3
Methionine	0.6	0.2	0.2	0.2	0.6	0.3	0.2	0.1	0.2	0.4
Isoleucine	2.0	1.5	1.5	1.4	1.7	2.2	1.5	1.1	1.6	1.2
Leucine	2.3	1.7	1.6	1.4	1.8	2.3	2.3	1.7	2.3	2.3
Tyrosine	1.0	0.7	1.0	0.8	1.1	0.8	1.0	0.7	1.0	0.9
Phenylalanine	1.3	0.6	0.8	0.9	1.0	1.1	1.1	0.7	3.2	1.1
Lysine	3.5	2.4	—	3.7	3.1	6.4	3.8	3.3	6.0	4.3
Histidine	2.2	2.4	1.2	2.2	—	2.5	1.3	2.2	2.6	4.0

Ingestion of blood meal (Table 4) resulted in concentration patterns similar, both as regards the decreases and maxima, to those observed after feeding overheated fish meal.

The presented results show that after ingestion of raw protein (fish meal or cracked corn) the plasma content of a great majority of amino acids decreased after 1 hr. and the highest values appeared after 3 hr. It should be noted that after feeding high-quality protein, such as fish meal, both the decreases and the maxima were more accentuated and they appeared practically simultaneously for almost all the amino acids determined. In the case of protein which had been submitted to

elevated temperature, either for the purpose of the experiment (overheated fish meal) or during technological processing (blood meal), differences between the individual amino acids were more marked, both as regards the time of appearance and the intensity of decreases and concentration maxima. In agreement with the observations of Long *et al.* [5] and Smith & Scott [12] that the addition of an amino acid affects the absorption of the amino acids derived from the ingested protein, the addition of lysine altered the concentrations of amino acids. Denton & Elvehjem [3] and Long *et al.* [5] reported that the amino acids added to the diet are absorbed more rapidly than the amino acids derived from protein. The same was observed in our experiments; the maximum for the added lysine was very distinct and appeared earlier than after ingestion of non-supplemented protein food (Fig. 1).

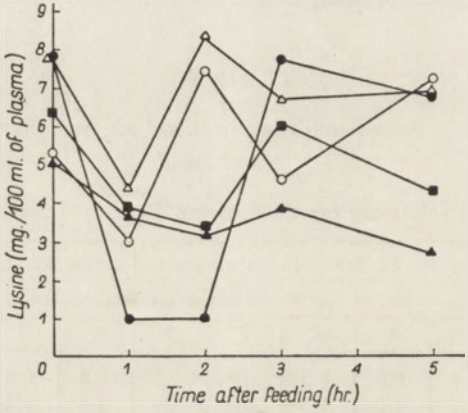

Fig. 1. Changes in the content of lysine in pig blood plasma after feeding: (●), fish meal; (○), fish meal with the addition of L-lysine; (▲), overheated fish meal; (△), overheated fish meal with the addition of L-lysine; (■), blood meal.

A very characteristic feature observed in the present experiments was the appearance of a decrease in the content of free amino acids in pig plasma after ingestion of different protein foodstuffs. A similar decrease was reported by Denton & Elvehjem [2] in the dog, but only after feeding zein. These authors believed that the decrease was due, at least in part, to the presence of carbohydrates in the diet. However, Longenecker & Hause [6] who kept dogs on a high-carbohydrate diet (e.g. wheat gluten with the addition of sucrose), did not observe any decline in plasma amino acid concentration.

The results presented in this paper seem to suggest that the time of appearance and the intensity of the decrease in plasma free amino acids depends, at least in part, on the composition of the ingested protein.

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ZMIANY W ZAWARTOŚCI WOLNYCH AMINOKWASÓW OSOCZA KRWI ŚWIŃ PO SPOŻYCIU POKARMU

Streszczenie

1. Oznaczano zmiany zawartości poszczególnych aminokwasów osocza krwi świń po spożyciu pokarmu zawierającego różne rodzaje białka; obserwowano obniżanie a następnie wzrost i osiąganie wartości maksymalnych w ciągu kilku godzin po karmieniu.

2. Skarmienie białka dobrej jakości powoduje wystąpienie silnie zaznaczonych spadków i wartości maksymalnych, przy czym i jedne i drugie występują dla prawie wszystkich oznaczonych aminokwasów w jednakowym czasie. Po skarmieniu białek o mniejszej wartości, spadki i wartości maksymalne zaznaczają się mniej zdecydowanie i występują w różnym czasie.

3. Dodatek L-lizyny do diety spowodował wcześniejsze pojawienie się silnie zaznaczonej wartości maksymalnej dla tego aminokwasu.

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SYNTHESIS OF URIDINE-3′-NAPHTHYLPHOSPHATE AND CYTOCHEMICAL LOCALIZATION OF RIBONUCLEASE BY THE AZO-DYE COUPLING TECHNIQUE

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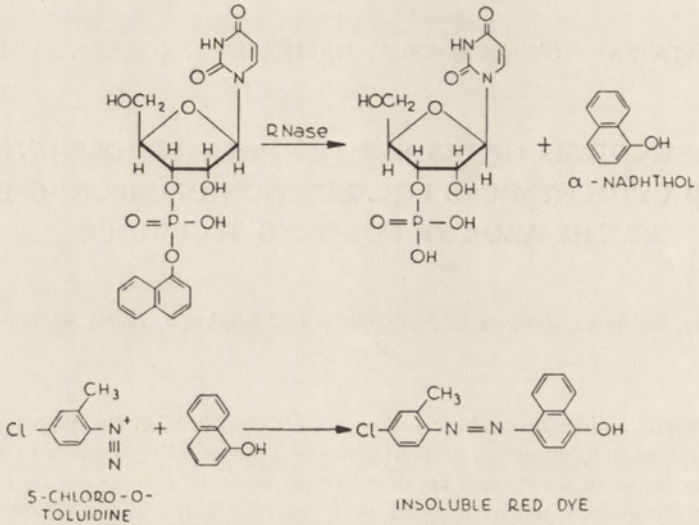
1. Several different synthetic procedures are described for the preparation of uridine-3′-naphthylphosphate from commercial uridine-2′(3′)-phosphate or uridine. The properties of the product are described. In particular it is specifically hydrolysed by ribonuclease to give uridine-2′:3′-cyclic phosphate and free naphthol. The rate of enzymic hydrolysis is about 100-fold greater than that for hydrolysis of uridine-2′:3′-cyclic phosphate. The synthetic procedure is also applicable to the preparation of uridine-2′-naphthylphosphate. 2. With the use of suitable diazotates in the azo-dye technique, uridine-3′-naphthylphosphate proved to be an excellent substrate for the cytochemical localization of alkaline ribonuclease in formalin-fixed tissue sections. It was also shown that formalin fixation, contrary to the reports of other observers, does not result in appreciable inactivation of ribonuclease in tissue sections, and possesses the pronounced advantage over other standard fixation methods in that formalin-fixed sections do not exhibit diffusion of the enzyme during the incubation periods employed. Localization patterns are illustrated for alkaline ribonuclease in a variety of rat tissues. 3. Preliminary trials demonstrated the feasibility of applying the above procedure for localization of acid ribonucleases.

The problems involved in the histo- and cytochemical localization of nuclease enzymes, viz. ribonucleases, deoxyribonucleases, phosphodiesterases, have been dealt with in detail elsewhere [14, 15, 17]. A procedure which appears to be quite specific and successful for one of the enzymes in this class, kidney phosphodiesterase (PDase I, see ref. [16])[1] was subsequently described. This was achieved by the use of the specific synthetic substrate α-naphthyl thymidine-5 -phosphate [16, 17] and was based on coupling of the enzymically liberated naphthol with a suitable diazotate according to standard azo-dye coupling techniques [9]. It was pointed out at that time that analogous α-naphthyl purine and pyrimidine ribonucleoside-2′(3′)-

[1] The following abbreviations are employed in this text: PDase I, kidney phosphodiesterase; PDase II, spleen phosphodiesterase; RNase, pancreatic ribonuclease; DNase, pancreatic deoxyribonuclease; DCC, dicyclohexylcarbodiimide.

-phosphates might serve as suitable cytochemical substrates for RNase-like enzymes, as shown in Scheme 1. Attention was further directed to the fact that such substrates would in all probability be highly specific because of the observation that α-naphthyl thymidine-3′-phosphate was quite inert to PDase II [16, 17], as well as PDase I.



Scheme 1

Note: the above diagram has been somewhat simplified. In accordance with the known transesterification reaction of pancreatic RNase, the initial products of enzymic hydrolysis are uridine-2′:3′-cyclic phosphate and naphthol. The former is subsequently slowly hydrolysed to uridine-3′-phosphate, but for all practical purposes this second reaction stage is of no importance here, as will be shown below.

One additional problem remained to be resolved, viz. the rather unexpected high rate of diffusion of RNases and DNases from the fresh-frozen and fixed tissue sections employed in previous investigations [14, 15]. Formalin fixation had not been employed in the foregoing studies because of the various reports in the literature on the deleterious effects of this reagent on ribonuclease activity [6, 7, 5]. We have now re-examined this question in some detail and have found that these reports are substantially in error. Formalin-fixed tissue sections do not, in fact, necessarily exhibit large losses in nuclease activity. Furthermore, the tendency for nucleases to diffuse from formalin-fixed sections is minimal. Preliminary trials with α-naphthyl uridine-3′-phosphate demonstrated that this compound was in many respects a suitable cytochemical substrate for pancreatic ribonuclease [18]. These findings, and additional information, including synthesis of the appropriate substrates, are presented in this report.

While the results outlined below indicate that our previously published procedure for the cytochemical localization of pancreatic RNase [14], based on the use of ribonucleoside-2′:3′-cyclic phosphates [13], could be substantially improved

by the use of formalin fixation, it will be seen from what follows that the employment of ribonucleoside naphthyl phosphates introduces considerable simplification and is more advantageous.

RESULTS AND DISCUSSION

Description of synthetic methods

It was decided at the outset of this study that the simplest and most convenient potential substrate (see *Concluding remarks*) would be uridine-3′-naphthylphosphate. Several different procedures were applied to its preparation, all of them based on the use of different blocking groups for the free carbohydrate hydroxyls. As will be seen from what follows, the choice of a suitable method was eventually dependent on the ease with which the blocking groups could be removed without decomposition of the final product. The methods employed were as follows:

(i) Uridine-3′-phosphate. prepared by the RNase hydrolysis of uridine-2′:3′--phosphate [13], was acetylated to give 2′,5′-di-*O*-acetyluridine-3′-phosphate. The phosphate group was then removed with the aid of prostate phosphomonoesterase to give 2′,5′-di-*O*-acetyluridine, which was condensed with the pyridinium salt of α-naphthylphosphate in the presence of DCC. However, when the resulting 2′,5′-di-*O*-acetyluridine-3′-naphthylphosphate was subjected to deacetylation, the products of hydrolysis were only uridine-3′-phosphate and free naphthol, even under the mildest possible conditions, pH 9.8 at room temperature.

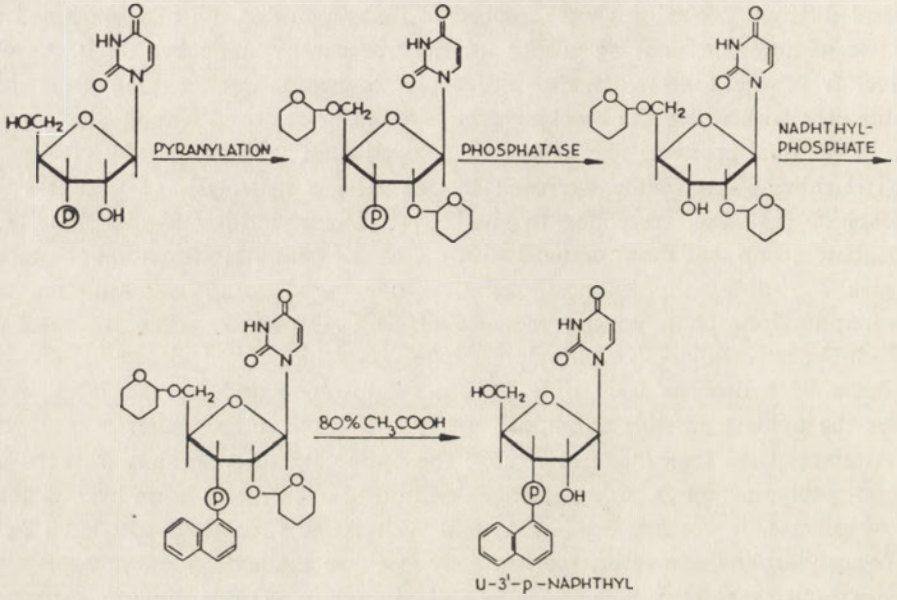
Attempts were then made to remove the protecting acetyl groups with the aid of non-specific esterases, using acetone-dried powders of rat or horse liver as sources of esterase. It was first established that such extracts readily deacetylated 2′,5′--di-*O*-acetyluridine. However, the relatively low esterase activity of these preparations, coupled with their high content of RNase, rendered this procedure ineffective on a preparative scale. It did, however, prove possible to isolate in this way a small sample of uridine-3′-naphthylphosphate, the identity of which was established by its hydrolysis by RNase to give uridine-3′-phosphate and free naphthol[2].

(ii) Several trials were devoted to preparing a mixture of the 2′ and 3′ isomers of uridine naphthyl phosphate by treatment of 5′-*O*-trityluridine with α-naphthylphosphoryl dichloride in pyridine-dioxan. However, when the resulting 5′-*O*-trityluridine-2′(3′))naphthylphosphate was subjected to the usual treatment for removal of trityl groups, 20 min. at 100° in 80% acetic acid, the only products were triphenylcarbinol, free naphthol and uridine-2′(3′)-phosphate. The same treatment at room temperature was quite ineffective for detritylation, and this procedure was therefore abandoned.

(iii) Attention was then directed to the use of dihydropyran as a blocking agent for the free carbohydrate hydroxyls, since the conditions subsequently required for their removal are much milder, viz. 80% acetic acid at room temperature. Uridine--3′-phosphate was treated with dihydropyran as described by Rammler & Khorana

[2] This result points to the utility of seeking a suitably active source of non-specific esterase for application in organic syntheses where particularly mild deacetylation conditions are called for.

[10] to give 2′,5′)di-*O*-tetrahydropyranyluridine-3′-phosphate. This product was dephosphorylated by means of prostate phosphomonoesterase; and the resulting 2′,5′-di-*O*-tetrahydropyranyluridine treated with an excess of the pyridinium salt of naphthylphosphate in the presence of DCC to give 2′,5′-di-*O*-tetrahydropyranyluridine-3′-naphthylphosphate, which was separated from the reaction mixture by paper chromatography. Removal of the pyranyl protecting groups in 80% acetic acid at room temperature gave the required uridine-3′-naphthylphosphate. The reaction scheme is illustrated in the flow diagram (Scheme 2).



Scheme 2

When uridine-2′(3′)-phosphate was treated with dihydropyran as above, and treated further as described below, the end product was identical (but in lower yield) with that obtained when the starting compound was uridine-3′-phosphate. This suggested that in a mixture of the two uridine phosphate isomers the 3′ hydroxyl was relatively inert to pyranylation, so that the products were 2′,5′-di-*O*-tetrahydropyranyluridine-3′-phosphate (R_F 0.44 in solvent *C*; 60% as estimated from an eluate of the spot) and presumably 5′-*O*-tetrahydropyranyluridine-2′-phosphate (R_F 0.30 in solvent *C*, 40%). Further experiments therefore utilized the commercially available uridine-2′(3′)-phosphate.

Subsequently it was found that the tetrahydropyranyluridine could be prepared directly from uridine, which resulted in further simplification of the procedure for preparation of the substrates.

Preparation of substrates

For paper chromatography (ascending technique) the following solvent systems were employed, as indicated: *A*, water-saturated butanol; *B*, ethanol - 1 M-ammo-

nium acetate (5 : 2, v/v); *C*, isopropanol - conc. ammonia - water (7 : 1 : 2, by vol.), using Whatman papers no. 1 and no. 3*MM*.

Attempted synthesis of α-naphthyl uridine-3′-phosphate via *2′,5′-di-O-acetyluridine-3′-phosphate*. Method of Rammler, Lapidot & Khorana [11] was employed for the preparation of 2′,5′-di-*O*-acetyluridin-e3′-phosphate. A neutral aqueous solution of this compound (10 mg./ml.) was brought to pH 5.8 with 0.1 M-acetate buffer and ineubated fro 12 hr. at 37° with an excess of prostatic phosphatase [12], following which chromatography in solvent *A* demonstrated the presence of 80% 2′,5′-di-*O*-acetyluridine (R_F 0.54), 10% 5′-*O*-acetyluridine)R_F 0i30) and 10% 5′-*O*-acetyluridine-2′:3′-phosphate (R_F 0.08).

The incubation mixture was brought to dryness under reduced pressure and the 2′,5′-di-*O*-acetyluridine selectively isolated by first extracting the residue with several 5-ml. portions of anhydrous methanol The methanol extract was then brought to dryness and the residue extracted several times with 5 ml, portions of dioxan, The dioxan was removed under reduced pressure and the residue of 2′,5′-di-*O*-acetyluridine dried over P_2O_5 under vacuum,

To 075 m-mole of 2′,5′-di-*O*-acetyluridine (246 mg,) dissolved in 7.5 ml. anhydrous pyridine was added 3.75 m-moles of the pyridinium salt of α-naphthylphosphate (840 mg. of free acid), followed by. 75 m-moles DCC (1.545 mg.) under anhydrous conditions. After 24 hr. at 29°, 7.5 m-moles pyridine in 3 ml. water was added with constant stirring. The reaction mixture was then evaporated to dryness under reduced pressure, dissolved in 7 ml. water and filtered. The filtrate was neutralized with hot saturated aqueous $Ba(OH)_2$, filtered and brought to dryness. The resulting α-naphthyl-2′.5′-di-*O*-acetyluridine-3′-phosphate (R_F 0.35 in solvent *A*) was separated from the bulk of naphthyl phosphate by extraction into dry acetone.

The acetone solution was evaporated to dryness and the desired product subjected to deacetylation in aqueous medium, brought to pH 9.8 with NH_4OH, for 12 - 48 hr. at room temperature. However, the only products resulting from this procedure were uridine-2′:3′-phosphate and free naphthol.

Attempts were then made to remove the acetyl protecting gronps with rat and horse liver acetone-dried powders [19], rich in esterase activity, by incubation of an aqueous solution of α-naphthyl-2′,5′-di-*O*-acetyluridine-3′-phosphate in 0.1 M-tris--HCl buffer, pH 7.4, with the acetone-dried powders. Paper chromatography demonstrated the appearance of some α-naphthyl uridine-3′-phosphate (R_F 0.23 in solvent *A*) and uridine-3′-phosphate. The former was identified by the colour reaction it gave with Fast Red TR after treatment with RNase, both on the chromatograms and in solution following elution from the paper. The latter was undoubtedly the result of partial degradation of α-naphthyl uridine-3′-phosphate by the RNase present in the acetone-dried powder preparations.

It is clear that the iow yield of substrate was due to the relatively weak activity of the esterase extracts as compared to their ribonuclease activity, as well as the rather high susceptibility of α-naphthyl uridine-3′-phosphate to ribonuclease (see below).

Attempted synthesis of α-naphthyl uridine-2′(3′)-phosphate via 5′-O-*trityluridine.* 5′-*O*-Trityluridine was prepared as described by Weimann & Khorana [20] and isolated from the reaction mixture according to the procedure of Michelson & Todd [8]. Four m-moles dry pyridine in 2.5 ml. anhydrous dioxan was added dropwise to a magnetically stirred solution of 1 m-mole 5′-*O*-trityluridine (486 mg.) and 2 m-moles α-naphthylphosphoryl dichloride (520 mg.) in 2.5 ml. anhydrous dioxan at room temperature. When the addition was terminated (about 1 hr.), stirring was continued for an additional 2 hr. following which 4 m-moles pyridine in 15 ml. water was added with continued stirring. Paper chromatography in solvent *A* demonstrated at this point the complete absence of trityluridine (R_F 0.9) and the appearance of two new products with R_F values of 0.34 and 0.56.

The reaction mixture was brought to dryness under reduced pressure and the residue taken up in chloroform, followed by decantation to remove insoluble material. The chloroform solution was then extracted first with one-fifth its volume of water, then with one-fifth its volume of a 1 M solution of pyridinium hydrochloride, pH 5.5, and finally brought to drynsse under reduced pressure. When the resulting gummy residue was subjected to detritylation by heating for 20 min. at 100° in 80% acetic acid, the products were found to include only triphenylcarbinol, free naphthol and uridine-2′(3′)-phosphate. Hydrolysis at room temperature was ineffective for removal of trityl protecting groups. Detritylation at intermediate temperatures was not considered promising and was not attempted.

α-Naphthyl uridine-3′-phosphate from uridine-3′-phosphate or from uridine-2′(3′)--phosphate, using tetrahydropyranyl protecting groups. Uridine-3′-phosphate was prepared from uridine-2′:3′-phosphate by means of digestion with RNase as elsewhere described [13,2], and was then treated with dihydropyran as described by Rammler & Khorana [10] to give 2′,5′-di-*O*-tetrahydropyranyluridine-3′--phosphate.

2′,5′-Di-*O*-tetrahydropyranyluridine-3′-phosphate was isolated on a semi--preparative scale by chromatography on Whatman 3*MM* with solvent *B*, and eluted with methanol. The eluate was brought to dryness under reduced pressure and the residue incubated in 0.1 M-acetate buffer, pH 5.8, with excess prostatic phosphomonoesterase for 24 hr. at 37°. Paper chromatography demonstrated 60% 2′,5′-di--*O*-tetrahydropyranyluridine (R_F 0.80 in solvents *A* and *C*), 30% 5′-*O*-tetrahydropyranyluridine (R_F 0.60 in solvents *A* and *C*) and 10% starting material (R_F 0.06 and 0.44 in solvents *A* and *C*). The incubation mixture was brought to dryness under reduced pressure, the residue extracted with methanol, the methanol extract filtered and evaporated to dryness, and the residue dissolved in 10 ml. water. The 2′,5′-di-*O*-tetrahydropyranyluridine was then selectively extracted from the aqueous solution with four 100-ml. portions of ether. The ether was evaporated off, the 2′, 5′-di-*O*-tetrahydropyranyluridine freed from impurities by preparative chromatography on Whatman 3*MM* with solvent *A*, followed by elution with methanol, and the alcoholic eluate brought to dryness. The residue was then further dried in P_2O_5 under vacuum.

To 1 m-mole 2′,5′-di-*O*-tetrahydropyranyluridine (412 mg.) in 10 ml. dry pyridine was added 5 m-moles of the pyridinium salt of α-naphthylphosphate (1.200 g.) and 10 m-moles DCC (2.060 g.). After 24 hr. at 29°, 10 m-moles pyridine in 5 ml. water was added with stirring. The reaction mixture was then taken to dryness at a temperature not exceeding 37°, following which 3 ml. water was added and the whole again brought to dryness. The residue was taken up in 10 ml. water and insoluble material filtered off. The filtrate was neutralized with hot saturated $Ba(OH)_2$, filtered, and taken to dryness under reduced pressure. The residue. α-naphthyl-2′, 5′-di-*O*-tetrahydropyranyluridine-3′-phosphate and naphthylphosphate) R_F values 0.56 and 0.10 in solvent *A*), was taken up in acetone and filtered. Chromatography on Whatman 3*MM* paper was then used to remove naphthylphosphate. The faster migrating band, which was entirely free from naphthylphosphate (no colour formation on spraying with a solution of prostate phosphomonoesterase containing Fast Red TR), was cut into strips and eluted with methanol. The methanol solution was brought to dryness and the product stored over P_2Os under vacuum to avoid hydrolysis of the pyranyl protecting groups. When required, a sample of the product was dissolved in water, shaken with Dowex-50 (H^+ form), filtered, and treated with 80% acetic acid at room temperature for 3 hr.t o effect removal of tetrahydropyranyl protecting groups. The solvent was removed under reduced pressure, and the product dried from aqueous solution.

α-Naphthyl uridine-3′-phosphate was found to be more stable in the acid form and was therefore stored as such over P_2O_5 under vacuum. The acid form was carefully neutralized with NaOH immediately prior to use. The product is readily identified on paper chromatograms (R_F values .023, 0.73 and 0.68 in solvents *A*, *B* and *D*) by the colour reaction following spraying with buffered RNase solution and then with aqueous Fast Red TR buffered to pH 9. In solution the product was completely hydrolysed by RNase to free naphthol and uridine-2′:3′-phosphate, the latter in turn undergoing enzymic conversion to uridine-3′-phosphate.

α-Naphthyl uridine-2′(3′)-phosphate from 5′-O-tetrahydropyranyluridine. 5′-*O*--Tetrahydropyranyluridine was phosphorylated as described above for 2′,5′-di-*O*--tetrahydropyranyluridine to give α-naphthyl-5′-*O*-tetrahydropyranyluridine-2′(3′)--phosphate, which was isolated from the reaction mixture by chromatography on Whatman 3*MM* with solvent *A* (R_F 0.37), followed by an additional short run with solvent *B*)R_F 0.90). Removal of tetrahydropyranyl protecting groups in acetic acid as above gave α-naphthyl uridine-2′(3′)-phosphate.

α-Naphthyl uridine-2′-phosphate. To an aqueous solution of α-naphthyl uridine--2′(3′)-phosphate (5 mg./ml.) in 0.05 M-tris-HCl buffer, pH 7.4, was added 50 μg./ml. srystalline RNase. Following incubation for 8 hr. at 20° the reaction mixture was chromatographed on Whatman 3*MM* paper with solvent *A*. The band corresponding to α-naphthyl uridine-2′-phosphate (R_F 0.23 as for the 3′ isomer) was then eluted with water.

α-Naphthyl uridine-2′(3′)-phosphate from uridine, using tetrahydropyranyl protecting groups. The tetrahydropyranyluridines may be prepared more conveniently directly as follows. To 1 m-mole (244 mg.) dry uridine in 5 ml. dimethyl sulpho-

xide is added 1 ml. trifluoroacetic acid and 6 m-moles dihydropyran. The reaction mixture is kept for 18 hr. at room temperature (20 - 25°). Approximately one-half the solvent is then evaporated off under reduced pressure at a temperature *not exceeding* 37°. The mixture is then cooled and 5 ml. concentrated ammonia added. The whole is then reduced to a small volume under reduced pressure and the products fractionated by chromatography on Whatman 3*MM* paper, using solvent *A*. Analysis showed 50% 5′-*O*-tetrahydropyranyluridine (R_F 0.61), 25% 2′,5′-di-*O*-tetrahydropyranyluridine (R_F 0.80) and 25% 2′,3′,5′-tri-*O*-tetrahydropyranyluridine (R_F 0.96). (N.B. This separation would undoubtedly be more effective on a larger scale by column chromatography).

The naphthylphosphates are prepared from these as above. From the 5′-*O*-tetrahydropyranyluridine one obtained a mixture of the 2′- and 3′-naphthylphosphates. From the 2′,5′-di-*O*-tetrahydropyranyluridine one may obtain exclusively the 3′-naphthylphosphate. Or, finally, one may treat the entire mixture, without fractionation, to obtain a mixture of the 2′- and 3′-naphthylphosphates.

Specificity and stability of substrate

Uridine-3′-naphthylphosphate, and presumably also the 2′ isomer, is slightly labile in aqueous medium, spontaneously decomposing with the liberation of free naphthol and uridine-2′:3′-cyclic phosphate. As mentioned above the free acid, when stored over P_2O_5 under vacuum, was more stable than the sodium salt. Immediately prior to use, the free acid is converted to the sodium salt by neutralization with NaOH or KOH. In neutral or slightly alkaline medium at room temperature only trace amounts of naphthol are liberated over a period of several hours, and these are readily removed by simply extracting the aqueous solution with ether.

If a large-scale preparation of the substrate is undertaken, it is desirable to terminate this following isolation of the 2′,5′-di-*O*-tetrahydropyranyluridine-3′-naphthylphosphate. This product is quite stable and smaller quantities can be depyranylated as desired in 80% acetic acid to give uridine-3′-naphthylphosphate, as described above.

Uridine-3′-naphthylphosphate is resistant to DNases and PDases I and II and is completely hydrolysed by RNase I to free naphthol and uridine-2′:3′-cyclic phosphate. Surprisingly the rate of enzymic hydrolysis of the naphthylphosphate is about 100-fold greater than that for uridine-2′:3′-cyclic phosphate, in agreement with the qualitative findings of Ehinger & Lagerstedt [5] for the relative rates of hydrolysis of cytidine-3′-benzylphosphate and cytidine-2′:3′-cyclic phosphate, but in sharp contrast to the results of Witzel & Barnard [21] with cytidine-3′-methyl and benzyl phosphates[3]. It should be emphasized that this extremely rapid rate of

[3] This rapid rate of hydrolysis of the naphthylphosphate by RNase has been confirmed by Witzel (personal communication) and the mechanism of this reaction will form the subject of a separate communication.

enzymic hydrolysis of the naphthylphosphate, which was quite unexpected, proved to be one of the key factors in the use of this substance for the cytochemical localization of RNase (see below).

Influence of formaldehyde on ribonuclease activity

The influence of formalin fixation on the ribonuclease activity of tissue sections calls for special comment in view of a number of reports indicating extensive inactivation in the presence of formalin of both crystalline ribonuclease [4] and tissue sections containing this enzyme.

A comparison was therefore made of the activity of tissue homogenates from pancreas fixed in Baker's formalin, as well as in formalin buffered at pH 7, with that of a control homogenate from fresh pancreas, all at a temperature of 4°. Using uridine-3′-naphthylphosphate and uridine-2′:3′-cyclic phosphates as substrates, the formalin-fixed homogenates showed no decrease in enzyme activity as compared to the fresh tissue. However, when activity was tested against yeast RNA as substrate, the fixed tissues exhibited only 20% of the activity of fresh material.

The foregoing results were further checked by the following procedure: crystalline RNase was preincubated in 2 M-formalin at 4° for 24 hr. and was then diluted 1000-fold ou addition to RNA, so that the formalin concentration in the reaction mixture was reduced to 0.002 M. Activity was tested against 1% RNA as substrate, incubation being in 0.1 M-tris buffer, pH 7.4, for 15 min. at 37°. When compared to a sample of RNase not previously treated with formalin, the activity was found to be decreased by only 10%. By contrast, when the formalin-treated enzyme was added to the reaction mixture so that the formalin concentration in the latter was 0.2 M, the measured activity was decreased to 8%.

The foregoing results, together with those obtained by cytochemical procedures (see below) clearly establish that formalin treatment at 4°, under conditions normally applied in histochemical procedures, does not inactivate RNase appreciably. The low degree of activity of RNase in formalin-treated tissue homogenates is due to the influence of the residual formalin on the RNA substrate.

Finally, when experiments analogous to the above were carried out with RNase subjected to formalin treatment at 20°, the enzyme was practically completely inactivated, in agreement with the results of Ehinger [4].

Cytochemical applications

Tissues were obtained from rats killed under ether anaesthesia. Sections were cut at a thickness of 20 μ from frozen material fixed in rormalin. Several trials were also run on paraffin 10 μ acetone-fixed sections. Although some attempts were made to localize RNase in 10 μ fresh frozen sections, the rapid rate of diffusion of the enzyme from such material [cf. ref. 14. 15] was such as to render the results of doubtful validity and they will not be touched upon here.

The incubation medium, 0.1 M-tris-HCl buffer, pH 9, contained 2 mg./ml. substrate and 4 mg./ml. Fast Red TR (Edward Gurr Ltd., London). This was found to provide approximately opitmal conditions for dye formation. It is true this pH is somewhat removed on the alkaline side from tha notrmally considered optimum for RNase activity, but it offers the advantage that the coupling reaction between enzymically liberated naphthol and the diazotate is quite rapid, so that diffusion of the azo-dye product is kept to a minimum. At pH 7.4, which is closer to the optimum for enzyme activity, the rate of coupling of the liberated naphthol was appreciably lower and resulted in marked diffusion of the precipitated dye.

Incubation at pH 9 was also accompanied by slight spontaneous decomposition of the substrate, with liberation of free naphthol. This gave with the diazonium salt a relatively slight precipitate which, however, was diffuse and only feebly adsorbed to the surface of the section; it was readily removed during the rinsing procedure after incubation. Its effect was considered negligible because of the short incubation times required, in view of the high rate of enzymic hydrolysis of the substrate.

Incubation times employed varied from 2 to 30 min. at room temperature for both Baker formalin and acetone-fixed sections. Controls consisted of sections inactivated by heating in aqueous medium at pH 9, or of normal sections incubated in the absence of substrate; these were always negative.

Baker formalin-fixed sections exhibited no evidence of enzyme diffusion. Even several hours rinsing with distilled water of such sections did not noticeably modify either the degree of localization or the magnitude of the enzyme activity (estimated visually). Furthermore, when a pancreas section (rich in RNase activity) was placed in contact with a liver section (the RNase activity of which is low) in the incubation medium, neither the activity nor localization on the pancreas section were affected, nor was there any increase in the amount of azo-dye complex on the liver section.

Preparations of glandular tissues fixed in acetone, alcohol or Carnoy solution, exhibited appreciable enzyme diffusion. For example, an acetone-fixed pancreas section possesses high RNase activity. However, immediately following immersion of such a section in the incubation medium, enzyme diffusion out of the section occurs. The result is a rapid coloration of the incubation medium itself, followed by the gradual deposition of the azo-dye complex over the entire section and even on the cover glass.

On the other hand, enzyme diffusion from acetone-fixed sections was found to be without effect on the localization of the enzyme in those areas of the section from which diffusion was either minimal or non-existent. This was best observed in acetone-fixed pancreas sections, the blood vessels of which contain RNase that does not diffuse out. Preincubation of such a section in buffer medium alone results in the removal of practically all enzyme activity from acinar cells, while the activity in the blood vessels apparently remains unaltered. When an acetone-fixed pancreas section was placed in contact, in the incubation medium, with another similar section which had previously been incubated in buffer alone, the end result was the appearance of azo-dye in the medium, with no modification of either the activity or localization of the enzyme in the preincubated section. A similar result was obtai-

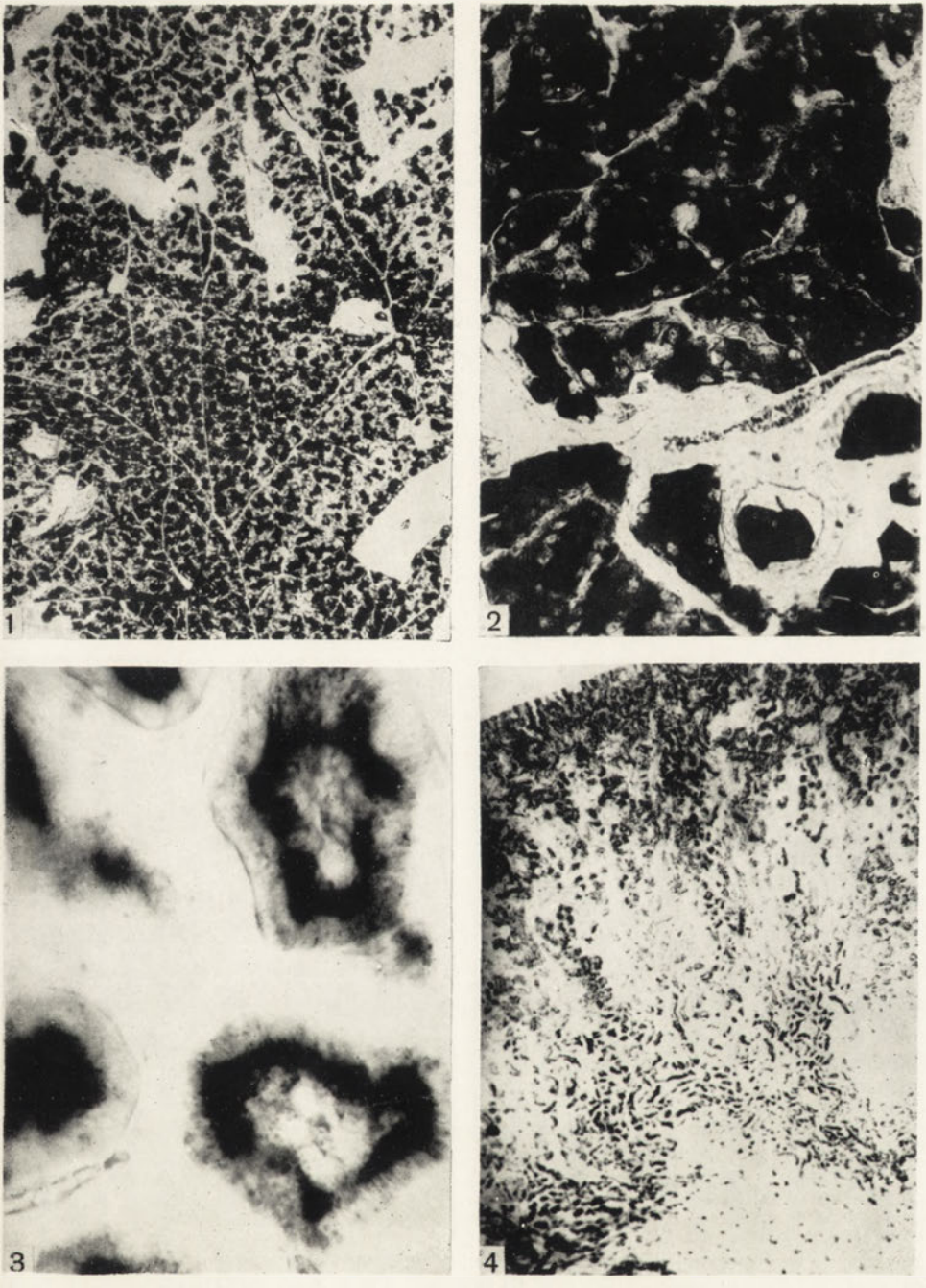
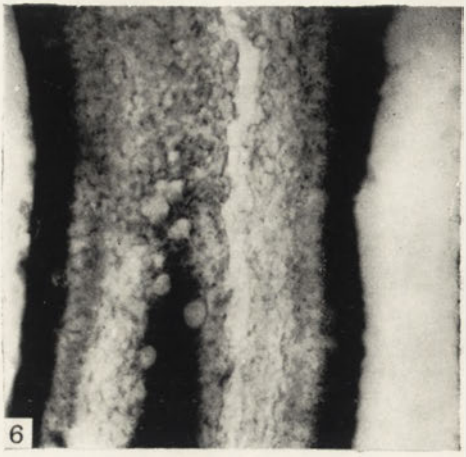
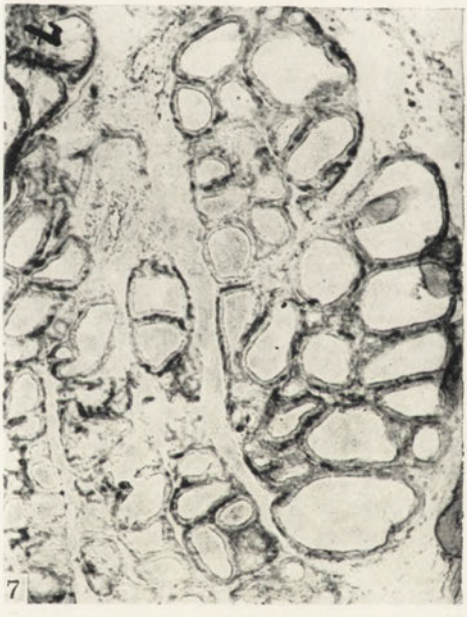


Plate I - 1. Pancreas (×80). Intense activity in acinar cells, islets negative, 2. Pancreas (×300). Nuclei and blood vessels negative, 3. Kidney (×800). High activity in brush border zone of proximal tubules, 4. Kidney (× 35). Cortex active, medulla and Malphigian corpuscles negative.

M. Zan-Kowalczewska, H. Sierakowska and D. Shugar (*facing p.* 246).





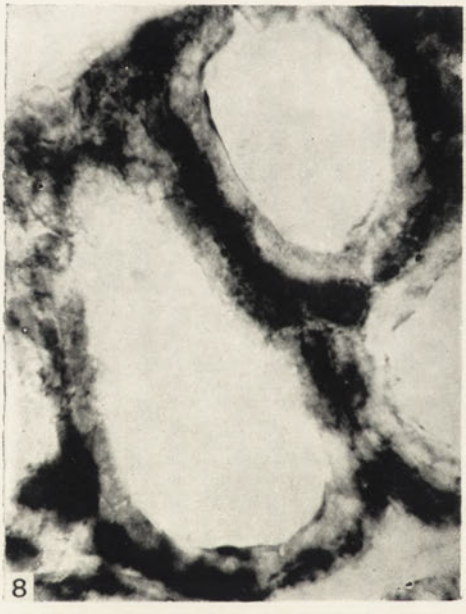


Plate II - 5. Duodenum (× 80). Intense activity in brush border, all other components negative, 6. Duodenum (× 320). Note positive granules in epithelial cells. 7. Thyroid (× 150). Small intercellular blood vessel walls show high activity, 8. Thyroid (× 800). Note absence of reaction in follicular cells, follicles and their secretion products.

ned when crystalline RNase was added to the incubation medium, demonstrating the absence of any specific adsorption of azo-dye complex in the sections.

Attempts to apply the above procedure to the localization of acid RNase, while only partially successful, appeared to be sufficiently promising to warrant further investigation. One of the attendant difficulties is the considerably lower activity of acid RNase, by comparison with that for the alkaline enzyme. In addition, at pH values above 5 there is some overlapping of activity with that for the alkaline enzyme. Finally, the coupling reaction of the liberated naphthol with diazotates is less efficient in the acid pH range.

A typical example of acid RNase localization is shown in Fig. 12 for kidney sections, following fixation in Baker formalin, according to the standard procedure of Davis & Ornstein [3] and Barka & Anderson [1]. The coupling diazotate in this instance was hexazonium *p*-rosaniline and several trials indicated that optimum coupling was at pH 5.7. Incubation was carried out with a substrate concentration of 2 mg./ml. In the pH range 5.0 to 5.6 there was considerable diffusion of the azo--dye product which made it impossible to obtain reasonably good localization patterns (however, see Addendum).

The localization pattern for acid RNase in kidney (Fig. 12) should be compared with that for localization of alkaline RNase in the same section (Fig. 3).

Attempts at localization of the acid enzyme in liver, spleen and pancreas were not too satisfactory, and further trials, involving also the use of other diazotates, are in progress.

Localization of RNase

The localization patterns observed in various formalin-fixed tissues are illustrated in Figs. 1 - 12, as follows:

Pancreas, 5 min. incubation (Figs. 1, 2). Enzyme activity appreciable in apical portions of acinar cells, with lower activity in the remaining cytoplasmic area of the acinar cells. The nuclei, islets of Langerhans, and the blood vessels are all negative. Some activity visible in the excretory ducts and the epithelium of the excretory ducts adjoining the lumen.

Kidney, 10 min. incubation (Figs. 3, 4). Enzyme activity in cortex but not in medulla. Proximal convoluted tubules positive with cytoplasmic activity concentrated in apical portions of cell and in the brush border zone. Nuclei, Malpighi corpuscles and other parts of nephron exhibit no activity. Positive reaction visible in large blood vessels, largely limited to their intima and adventitia.

Duodenum, 10 min. incubation (Figs. 5, 6). Surface of villi covered with heavy dye deposits. Apical portions of epithelial cells exhibit distinct positive microgranules. Some of the epithelial cells of the mucosal glands show an apical reaction. All other components of the duodenum consistently negative.

Thyroid, 20 min. incubation (Figs. 7, 8). Walls of interfollicular capillary blood vessels stain intensely. Follicular epithelium and the follicular contents are negative.

Skin (foot pad), 30 min. incubation (Fig. 9). Note positive reaction in small blood vessels immediately underlying corium. Both peripheral layer of epithelial cells, and free cell surface of sweat glands, also positive.

Submaxillary gland, 30 min. incubation (Fig. 10). Distinct enzyme activity in cytoplasm of serous acini, salivary ducts and small blood vessels. Nuclei, mucous acini, striated ducts, veins and arteries all negative.

Trachea, 20 min. incubation (Fig. 11). Cells of perichondrium intensely active. The walls of the lacunae are also stained heavily. Submucosal glands positive. Epithelial cells immediately surrounding the organ's lumen are negative.

Kidney, acid RNase pH 5.7, 25 min. incubation (Fig. 12). Positive reaction can be observed only in the cortex portion, with dye formation in proximal convoluted tubules where the reaction is most pronounced in the parts of cytoplasma adjacent to the lumina. All other structures are negative.

Concluding remarks

From a general point of view, it is of considerable interest that formalin fixation does not appreciably inactivate RNase activity in formalin-fixed sections, while at the same time such sections do not exhibit the extensive diffusion of enzyme previously observed with other methods of fixation [14, 15]. This opens the possibility for more extensive investigations of the precise intracellular localization of RNase, and their extension to material in which normal cellular functions have been disturbed, e.g. in various pathological states. In view of the foregoing, attempts are under way to further improve the synthetic methods for uridine-3′-naphthylphosphate outlined above.

There are, however, two points which are somewhat puzzling. In view of the findings demonstrating that formalin fixation procedures at 4° do not markedly affect RNase activity, it is difficult to understand the variance in localization obtained following formalin or acetone fixation. For example, following formalin fixation there is a complete absence of RNase in the pancreas blood vessels, while at the same time the acinar cells are strongly positive, with no evidence of diffusion. With acetone fixation, on the other hand, the pancreas blood vessels are positive, while the acinar cells exhibit the same activity, somewhat diffuse. It is, of course, conceivable that there is more than one alkaline RNase behaving differently towards different fixatives; or that there is only one enzyme, which is bound differently to different structures.

The second point which requires further clarification is the failure to observe any positive reaction in either liver or spleen, both of which are known to contain alkaline RNase. This is all the more curious in that attempts to detect the enzyme in fresh frozen sections of these tissues were equally unsuccessful. This problem is at present the subject of further study.

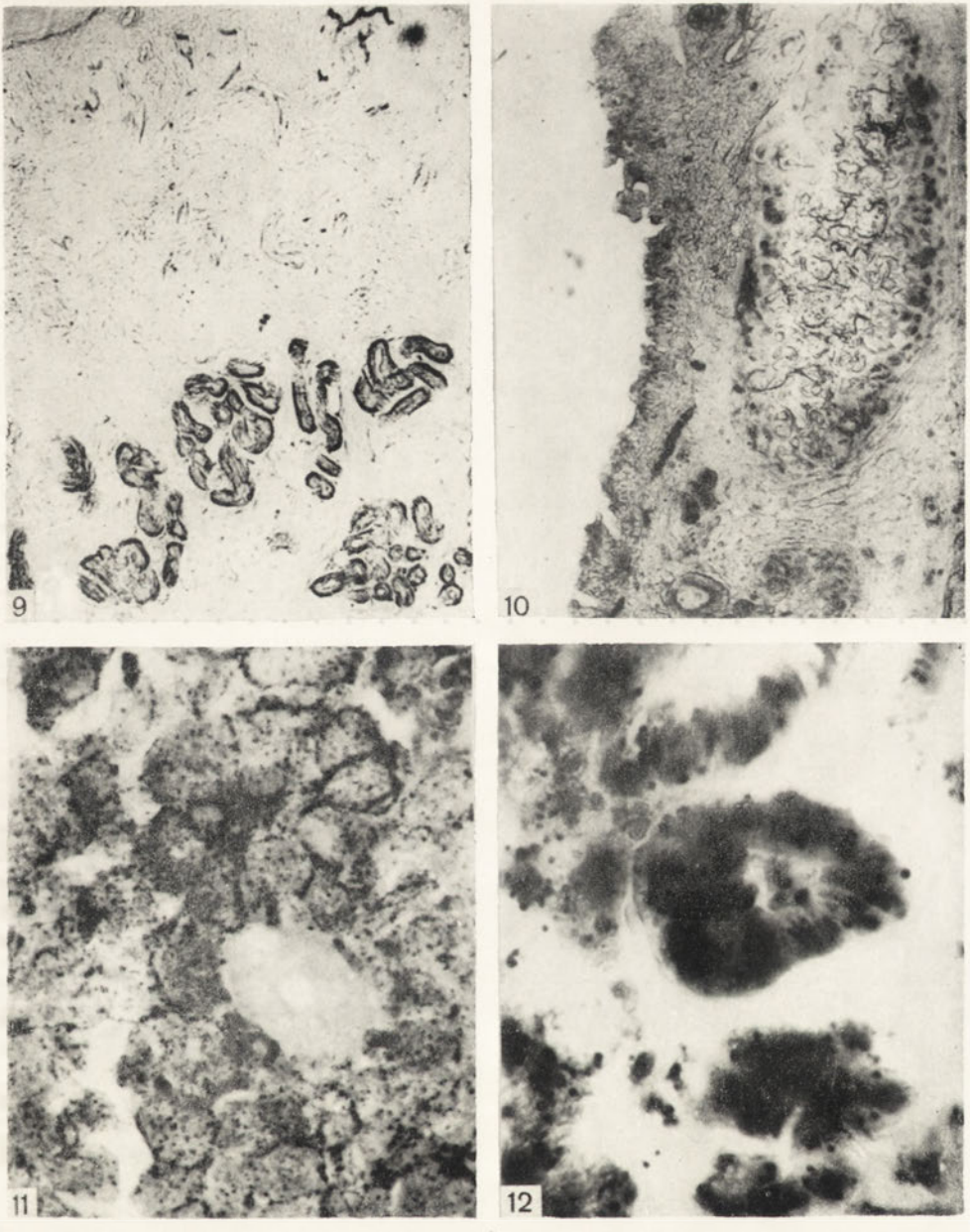


Plate III - 9. Food pad (× 80). Small blood vessels and sweat glands positive, 10. Trachea (× 18). Intense activity in submucosal glands and walls of lacunae, 11. Submaxillary gland (× 540). Serous cells positive, 12. Kidney (× 800); acid RNase. Activity in cytoplasm of proximal tubules (cf. Fig. 3).

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SYNTEZA 3'-NAFTYLOFOSFORANU URYDYNY I ZASTOSOWANIE GO DO CYTOCHEMICZNEJ LOKALIZACJI RYBONUKLEAZY PRZEZ SPRZĘGANIE Z SOLAMI DWUAZONIOWYMI

Streszczenie

1. Opisano kilka różnych metod syntezy 3'-naftylofosforanu urydyny oraz 2'-naftylofosforanu urydyny z handlowego preparatu 2'(3')-fosforanu urydyny lub urydyny. Przebadano i opisano własności produktu. Otrzymany preparat jest specyficznie hydrolizowany przez rybonukleazę do

2′ : 3′-cyklicznego fosforanu urydyny i wolnego naftolu. Szybkość enzymatycznej hydrolizy substratu jest około 100 razy większa niż hydroliza 2′:3′-cyklicznego fosforanu urydyny.

2. Przy zastosowaniu odpowiednich soli dwuazoniowych do sprzęgania uwolnionego enzymatycznie naftolu, 3′-naftylofosforan urydyny okazał się odpowiednim substratem dla lokalizacji alkalicznej RNazy w skrawkach tkanek utrwalanych w formalinie. Ponadto w przeciwieństwie do innych autorów wykazano, że utrwalanie w formalinie nie inaktywuje RNazy, a także zapobiega dyfuzji enzymu ze skrawków w czasie prowadzenia inkubacji. Przedstawiono przykładowo rozmieszczenie rybonukleazy alkalicznej w różnych tkankach szczura.

3. Wstępne doświadczenia wykazały, że powyższą metodę można również zastosować do cytochemicznej lokalizacji rybonukleazy kwaśnej.

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Addendum. Since submission of the above manuscript, we have further examined the question of localization of acid RNase in various tissues. The results indicated that what was originally presumed to be a positive reaction for acid RNase in kidney (Fig. 12) was, in fact, due to alkaline RNase, the activity of which is appreciable even at pH 5.7. The apparent more extended localization of the RNase at acid pH appears to be due to the fact that hexazonium *p*-rosaniline is a more effective coupling agent under these conditions and is also less inhibitory towards RNase than Fast Red TR.

We have, on the other hand, shown that, in the absence of alkaline RNase, one may readily localize acid RNase. This was established by the use of fresh sections of pea leaves, which are known to contain only acid RNase. It follows that localization of acid RNase in tissues which contain also alkaline RNase will require some supplementary techniques for differentiating the two enzymes. This is the subject of further study.

Finally, we have now succeeded in detecting and localizing alkaline RNase in rat liver. The positive reaction obtained was limited to the parenchymal cell membranes lining the bile canaliculi. This will be elsewhere described.

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INTERACTION OF POLYRIBOTHYMIDYLIC ACID WITH METAL IONS AND ALIPHATIC AMINES

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1. The effect of mono- and divalent metal ions on the formation and thermal stability of the ordered state of polyribothymidylic acid was investigated. It was demonstrated that the interaction of monovalent ions with the polymer is weak, whereas divalent ions are strongly bound to the polymer. 2. Similarly, the interaction was investigated of a number of mono-, di- and polyamines with polyribothymidylic acid, various homopolyribonucleotides, as well as with some complementary homopolymer pairs. 3. Prerequisites were determined for the appearance of an additional stabilizing effect induced by some di- and polyamines. For instance, maximal stabilization afforded by putrescine and cadaverine is manifested toward a polynucleotide capable to form a regular ordered structure, and containing thymine in both strands. A possible mechanism for the appearance of this specific effect is discussed in relation to model and natural polynucleotides.

Investigations of the interaction of natural and synthetic polynucleotides with metal ions and some aliphatic amines have attracted recently considerable interest. It is hoped that these studies will provide additional information on details of polynucleotide structure as well as on forces responsible for helix stability. The present communication describes the influence of these cations on the secondary structure of polyribothymidylic acid[1]. This ribonucleotide homopolymer was chosen as a model for a number of reasons. It is known that poly-rT is highly ordered at room temperature in the presence of Mg^{2+} ions. Its ordered form - random coil transition, which is fully reversible, occurs within a very narrow temperature range

[1] Abbreviations used in this text: poly-A, polyadenylic acid; poly-U, polyuridylic acid; poly-rT, polyribothymidylic acid; poly-C, polycytidylic acid; poly-5MeC, poly-5-methylcytidylic acid; poly-I, polyinosinic acid.

Poly-(A+U) is the twin-stranded complex of poly-A and poly-U, with similar connotations for the other polymers; d as a prefix refers to the corresponding deoxy polymer, e.g. poly-dI is polydeoxyinosinic acid; poly-dAT, alternating copolymer of dA and dT.

T_m is the temperature at which half of the final change in optical density on heating has taken place.

Δt is the change in temperature required to bring the optical density transition from 10% to 90% completion.

testifying to a co-operative process, and is accompanied by a 50% hyperchromic effect [28]. Poly-rT possesses an unusually high intrinsic viscosity, similar in magnitude to that of denatured DNA, and a buoyant density of 1.547 in cesium sulphate which is less than that of poly-rAU and also lies in the neighbourhood of synthetic DNA's rather than RNA's [23]. Taken in conjunction all these properties, rather unique among synthetic ribonucleotide homopolymers, make poly-rT a suitable model for studying the conditions of the helix-coil transition, the more so that the transition itself occurs in the vicinity of room temperature. Changes in T_m and Δt of poly-rT as a function of the concentration of certain mono-, and divalent metal ions, mono-, di-, and polyamines are presented along with comparative experiments on the stability of poly-U, and the poly-(A+U) and poly-(A+rT) 1 : 1 complexes, and discussed relative to the structure of natural nucleic acids. A preliminary report of part of these investigations has been published [25].

EXPERIMENTAL

Poly-rT was prepared as previously described by the actoin of polynucleotide phosphorylase from *Azotobacter vinelandii* on 5′-methyluridine-5′-pyrophosphate [28]; its physical characteristics have been reported [23]. Poly-A and poly-U employed were commercial samples (Miles Chemical Co., Clifton, N.J.); they were further purified prior to use by deproteinization and exhaustive dialysis, finally against redistilled water.

Analytical grade reagents were used throughout. Unbuffered solutions of metal salts and amine chlorides were employed, unless otherwise indicated. Metal chlorides were used in all instances, except for Cu^{2+}. $CuCl_2$ behaved much like a monovalent cation and $CuSO_4$ was used instead. In the case of hygroscopic metal chlorides, e.g. $MgCl_2$, the concentration of a stock solution was checked by chloride ion titration. The various amines employed were recrystallized, when available as hydrochlorides; when available as free amines they were distilled, neutralized with HCl and recrystallized from an acetone-ethanol solution. Salt solutions were prepared in glass-redistilled water and saline solutions of polymers were obtained either by addition of samples of concentrated salt solutions directly to the spectral cuvettes or by exhaustive dialysis of the polymer against the salt solution. All experiments were carried out at polymer concentration of $2.0\text{-}3.5 \times 10^{-5}$M unless otherwise described in the text.

Temperature profiles were run in a Unicam SP 500 spectrophotometer equipped with a specially constructed 3-cuvette brass block compartment, the temperature of which was controlled by means of a water-glycerol mixture from a Hoeppler ultrathermostat. A thermistor in a dummy cuvette served as temperature indicator. The calibrated thermistor-Wheatstone bridge assembly enabled temperature readings with an accuracy of 0.2° up to a temperature of 75°. Hilger stoppered cuvettes were employed, and no corrections were made for thermal expansion of the liquid. The temperature profiles shown in the figures are all reversible and gave the same points when subjected to a second heating cycle.

RESULTS

Monovalent metal ions. Saline solutions of poly-rT form an ordered structure which can be melted out by heating and completely reversed on cooling. The formation of an ordered structure in the presence of monovalent ions is observed at salt concentrations exceeding ~1000-fold that of the polymer. This behaviour of monovalent cations contrasts sharply with that of divalent cations by exhibiting a poor affinity to the polymer, and more specifically, to the phosphate binding sites [5, 4]; it was therefore termed "weak interaction" by Felsenfeld & Huang [5]. Figure 1 presents a set of optical density melting curves typical for a monovalent cation, obtained with poly-rT at various NaCl molarities in the range 0.05 - 1.0 M.

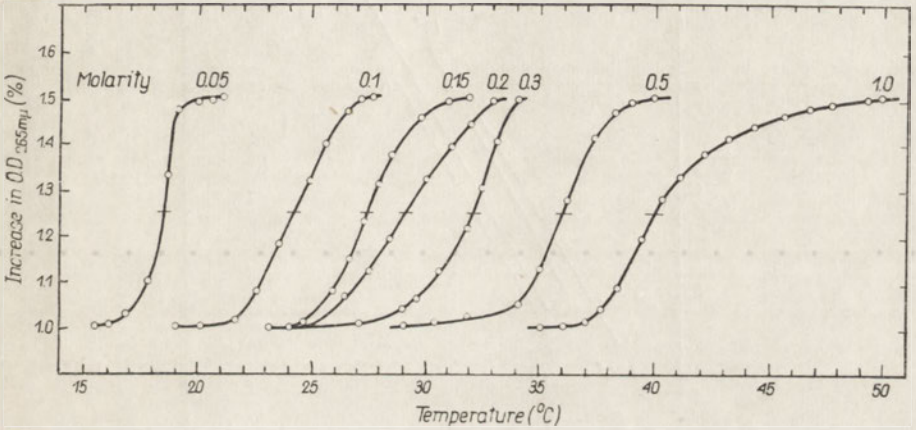

Fig. 1. Melting profiles for poly-rT in the presence of various concentrations of Na^+ as indicated.

It will be noted that the curves are asymmetric and Δt increases with an increase in Na^+ molarity while the overall hyperchromicity remains unchanged. Curves of almost the same shape were obtained with LiCl and KCl though same minor differences in T_m were observed (see Fig. 2); Li^+ was found to be the most effective among this class of ions followed by Na^+ and K^+. It is known that T_m of native DNA increases linearly with a decrease in monovalent metal ion radius [31, 8]. The increase of the width of the transition of poly-rT with a raise in salt concentration (cf. also Fig. 3) is in contrast to the behaviour of DNA and some of the double-stranded 1:1 complexes of complementary polynucleotides [3]. It is, however, analogous to the behaviour of synthetic alternating sequence copolymers of the poly-dAT [15] or poly-rAU type [1]. The re-formation of the ordered state of these polymers appears to proceed with a first-order rate. This indicates that strand collision is not a rate-limiting factor and the polymers possess an intrinsic capability to form intramolecular ordered states. In connection with the foregoing it should be emphasized that experimental conditions for measuring the rate of re-formation of the ordered state of poly-rT could not be found. At any salt concentration, whether with mono- or divalent cations or amines, at which the ordered state could be formed, at least 70% of the total decrease in optical density of the ordered state

versus coil occurred instantly, either on addition of a concentrated salt solution to the polymer in water or on chilling of the "melted out" form below T_m.

Figure 2 reveals that the dependence of T_m of poly-rT on the logarithm of salt concentration is linear as it is found for all the other ordered states [15, 1, 14]. Furthermore, while poly-rT is much less stable than the 1 : 1 complexes of complementary polynucleotides, the slopes of the corresponding plots of T_m values *versus*

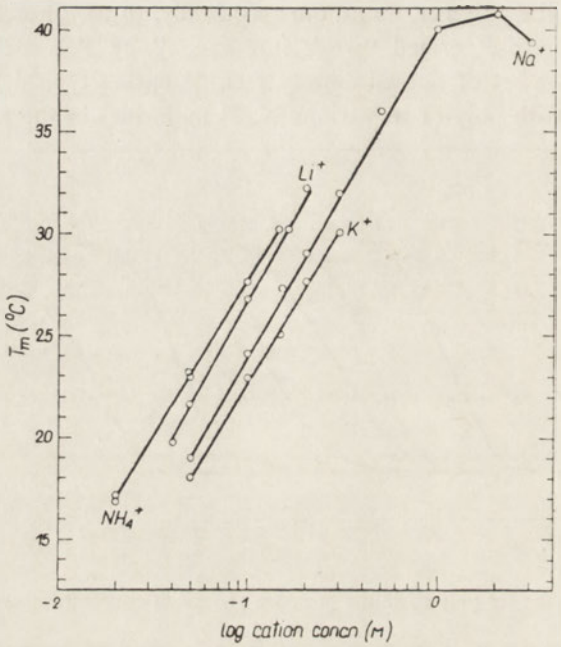

Fig. 2. Variation of T_m of poly-rT with the concentration of monovalent metal ions as indicated. Profiles were run by following optical density changes at 265 mμ. NH_4^+ curve is inserted for comparison.

log M_{Na} are rather similar in all cases (cf. Fig. 6). It is difficult to assess whether this is coincidental or whether it indicates the existence of a double-stranded structure in poly-rT. It was suggested that the greater slope of the corresponding curve for poly-dI might be related to the three-stranded complex which it apparently forms [14].

At Na^+ (K^+, Li^+) concentrations inferior to $2 - 3 \times 10^{-2}$ M one observes a rather labile, partly organized state of poly-rT. When a cold solution of the polymer at a concentration within the range 5 - 10 mg./ml. is diluted into a spectrophometer cuvette at 10°-15° to a concentration of about $3 - 5 \times 10^{-5}$ M, the extinction is initially quite low and variable. If such a solution is left at room temperature for a few hours or vigorously shaken, the extinction rises to the normal value for the coil form. If this solution is now stored in the refrigerator, the extinction decreases over a period of weeks, but does not return to the final value typical for the ordered state. This tendency to form "aggregates" in concentrated solution (the solution

exhibits no signs of turbidity by measurements at 340 mμ), with subsequent behaviour in dilute solution, was not observed for poly-U, nor for poly-A or poly-C; but a somewhat analogous behaviour is shown by poly-5-MeC [27]. Upon addition of poly-A to a partly organized form of poly-rT in a spectrophometer cuvette the "aggregation" disappears instantaneously with concomitant formation of poly-(A+rT).

At Na^+ concentrations above 1 M the logarithmic plot levels off and then the T_m values gradually decrease (Fig. 2). At 3 M-Na^+ the ordered form still exists, if pre-formed at lower molarity, but on melting out it cannot be reversed during a 21 day storage in refrigerator. The denaturing effect of Cl^- at very high concentration on DNA was described by Hamaguchi & Geiduschek [9], the Cl^- ion acting as a hydrophobic-bond breaker.

The experiments described, as well as the following ones, were carried out in unbuffered solutions in order to avoid any additional factors which could affect T_m, e.g. any ions required for pH adjustment. In turn, it became necessary to examine the effect of pH in the range of about 4 - 8 on the thermal transition of poly-rT. The results of the corresponding experiments are presented in Table 1; it appears that pH changes within the range tested affect neither T_m nor the shape of the transition curves if Cl^- remains the predominant anion in solution; the three heating curves in unbuffered or slightly buffered 0.15 M-NaCl solutions were virtually indistinguishable. Further experiments in which acetate buffers were employed at the same Na^+ molarity indicate that it is rather the presence of the acetate ion which influences slightly the transition.

Table 1

Comparison of effects of buffered and unbuffered solutions on the thermal transition of poly-rT at 265 mμ

Buffer	NaCl added to final concn. (M)	pH	T_m (°C)	Δt (°C)
None	0.15	5.6	27.3	5.5
0.005 M-Na-phosphate	0.15	7.8	27.3	5.3
0.005 M-Na-acetate	0.15	4.2	27.2	5.3
0.15 M-Na-acetate	—	5.5	26.2	5.8
0.15 M-Na-acetate	—	4.0	25.5	5.5
0.15 M-Na-acetate	—	3.0	25.4	6.1

Divalent metal ions. Poly-rT interacts strongly with divalent ions and the formation of the ordered state is completed, as may be judged from total optical density decrease, when about one equivalent of divalent ion is present per mole of polymer phosphate (Fig. 3). The strong type of interaction is further supported by the fact that 100% reversibility occurs instantly on addition of a concentrated salt solution

to the polymer in water or on chilling the coil form tenths of a degree below T_m; only at concentrations above 0.1M salt the immediate reversibility amounts to 70%, the full decrease in optical density occurring within minutes at room temperature.

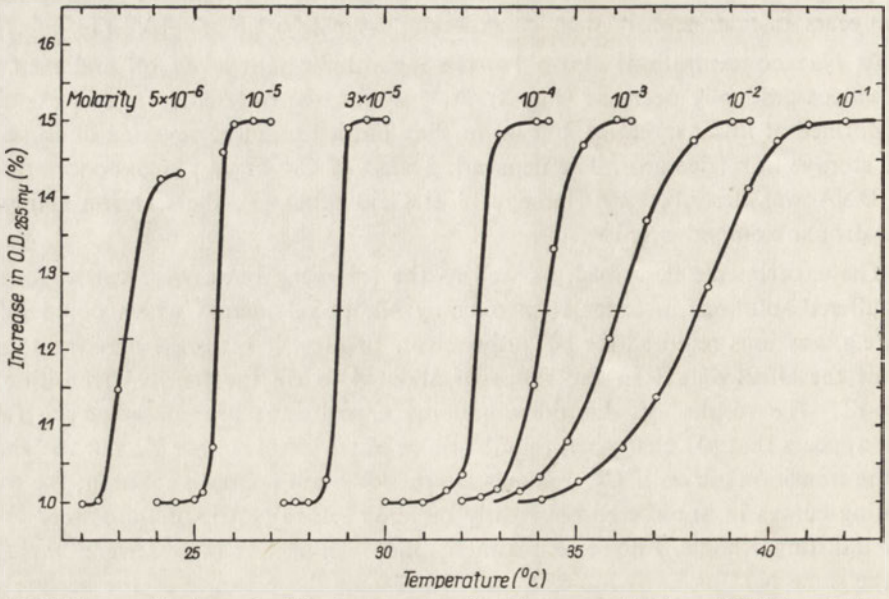

Fig. 3. Melting profiles for poly-rT in the presence of various concentrations of Mg^{2+} as indicated.

Figure 3 presents a set of heating curves typical for a divalent cation obtained with poly-rT at various $MgCl_2$ molarities in the range 5×10^{-6} - 10^{-1} M. Again, as with monovalent cations, Δt increases with an increase in salt concentration with one notable exception of very low molarities within the range 5×10^{-6} - 8×10^{-6} M-$MgCl_2$, i.e. when the solution contains less than one equivalent of Mg^{2+} per mole of phosphate (cf. Fig. 5). It will be noted that in this case the hyperchromicity does not reach its plateau of 50%. At salt concentrations inferior to 5×10^{-6} M the system behaves as it was described for low Na^+ concentrations and the appearance of the labile, partly ordered state is observed.

It is seen from Fig. 3 that the presence of divalent ions enhances the co-operativeness of the transition. The corresponding Δt values for 10^{-5}, 10^{-4}, 10^{-3}, 10^{-1} M-$MgCl_2$ are, respectively, 0.3°, 0.6°, 2°, 3.6°, whereas the lowest Δt in the presence of NaCl is 2.8° at 5×10^{-2} M.

At very high $MgCl_2$ concentrations T_m values gradually decrease and the heating curves become irreversible, as was the case for high NaCl content. This is the only point where the two logarithmic plots of T_m *versus* Mg^{2+} and Na^+ cross each other (cf. Figs. 2 and 4).

With the class of divalent metal ions it was possible to study the transition over a 10^6-fold increase in salt molarity. In accord with the former results with monovalent cations the variation of T_m is linear with the logarithm of $MgCl_2$ but the slope of the corresponding curve is much smaller. Actually, two different slopes are evident:

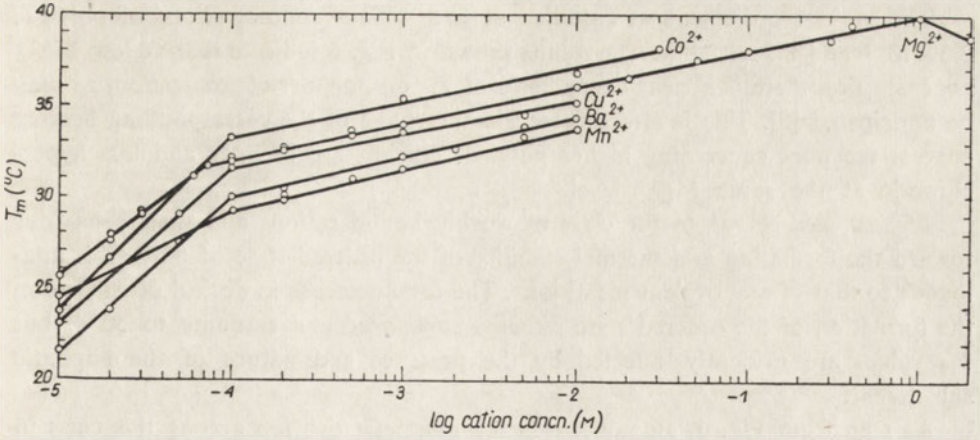

Fig. 4. Variation of T_m of poly-rT with the concentration of divalent metal ions as indicated. Profiles were run by following optical density changes at 265 mμ.

the initial greater one, for the "unsaturated" system (until there are about 5 equivalents of divalent ion per phosphate), followed by a second smaller slope for the already "saturated" system. The rather definite change of slope (cf. also Fig. 5) may be taken to imply a higher degree of packing of the aromatic rings in the helix, and, subsequently, a diminished extent of exposure to water. The behaviour of

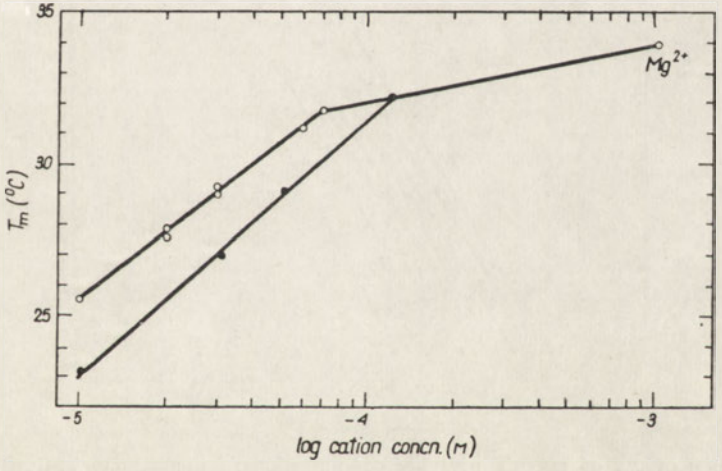

Fig. 5. Dependence of T_m of poly-rT on its molarity (as monomer) at low Mg^{2+} concentration. (○), 2.5×10^{-5} M, (●), 8.5×10^{-5} M.

poly-rT in the presence of other divalent ions tested: Mn^{2+}, Ba^{2+}, Cu^{2+}, Co^{2+}, was very similar to that in the presence of Mg^{2+} in terms of both T_m and Δt; Co^{2+} was found to be the most effective among this class. In connection with the recent reports [2, 12, 29] on the specific effect of Cu^{2+} on DNA, apparently because of its complexing with some of the bases [2], it should be pointed out that no differences whatever were found in the presence of up to 10^{-3} M-Cu^{2+}.

The Mg^{2+} requirement was studied at two various concentrations of poly-rT, 2.5×10^{-5} and 8.5×10^{-5} M. The results shown in Fig. 5 indicate that at low Mg^{2+} concentration there is a clear dependence of T_m on polymer concentration, as may be anticipated [5]. This is also reflected in the shape of the corresponding heating curves: the ones concerning higher polymer content are broader and less hyperchromic at the same Mg^{2+} level.

Monoamines belong to the class of weakly bound cations and their behaviour toward the formation and thermal stability of the ordered state of poly-rT is analogous to that of monovalent metal ions. The total decrease in optical density upon the formation of the ordered state remains unchanged and amounts to 50%, but T_m values are evidently affected by the presence and nature of the aliphatic substituent.

As seen from Fig. 6, ammonium is the most effective ion among this class in rising T_m; any substitution tends to decrease the structure preserving ability. An

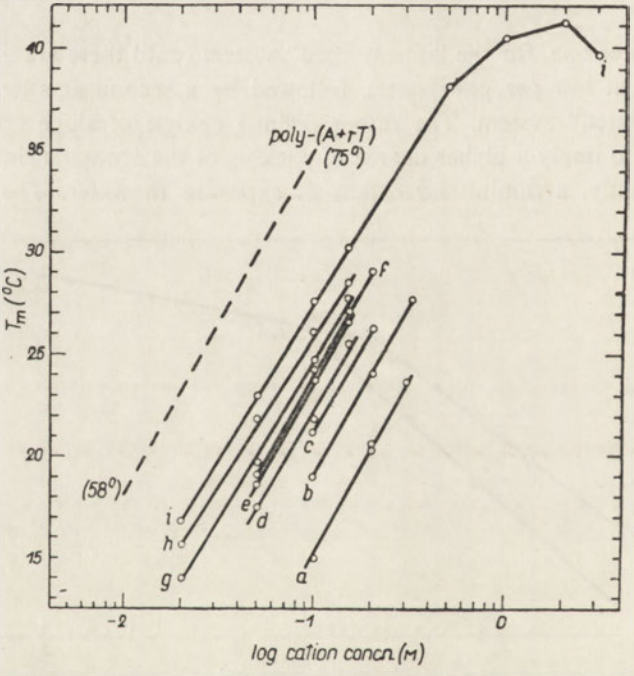

Fig. 6. Variation of T_m of poly-rT with the concentration of monoamine hydrochlorides: *a*, triethylamine; *b*, *n*-butylamine; *c*, *n*-propylamine; *d*, trihydroxymethylaminomethane; *e*, triethanolamine; *f*, trimethylamine; *g*, ethylamine; *h*, methylamine; *i*, NH_4^+. Profiles were run by following optical density changes at 265 mμ. Curve for poly-(A+rT) is inserted for comparison (taken from ref. [27]). Note different temperature scale for the latter.

elongation of the alkyl chain decreases T_m; e.g. from 26.1° for methylamine to 19.1° for buthylamine, both at 0.1 M. Di- and tri-substituted amines are more effective structure breakers than the corresponding mono-substituted amines and T_m drops from 24.8° for ethylamine to 15.1° for triethylamine, both in 0.1 M solutions.

A branched chain as in isopropylamine (not shown in Fig. 6) has a negative effect on T_m as compared to propylamine. On the other hand, triethanolamine proved to be more effective in maintaining helix stability than the corresponding triethylamine, and tris-HCl buffer is almost as effective as the former. It appears that the substitution of a hydrophilic group increases T_m in this case (see Discussion).

Venner, Zimmer & Schröder have investigated the thermal transition of native DNA in saline solutions in the presence of various alkyl monoamines and have found similar regularities [30].

Di- and polyamines. Certain widely distributed di- and polyamines are known to possess high affinity for binding polynucleotide phospate [13, 4]. It was demonstrated that diamines NH_2-$(CH_2)_n$-NH_2 and spermine exhibit a specific effect on the thermal stability of native DNA, but not RNA. This effect is positively correlated with the AT content of DNA; cadaverine (n=5) was shown to be more effective than the lower and higher diamines studied [19, 18]. On the other hand, diamines do not exhibit any *specific* effect, as compared to divalent metal ions, on T_m of the

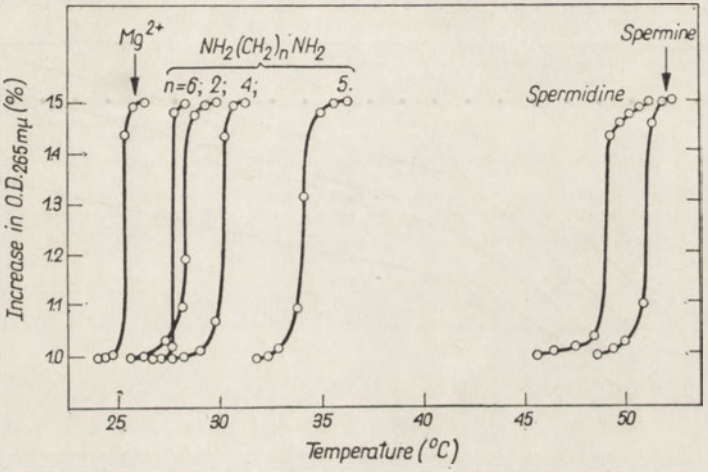

Fig. 7. Melting of poly-rT in the presence of Mg^{2+} and amines, as indicated in the diagram. Polymer concentration 4×10^{-5} M, as monomer, and equivalent counterion concentration. Identical curves were obtained in redistilled water and in 10^{-2} M-phosphate buffer, pH 7.4.

1 : 1 complexes of poly-(A+U), poly-(I+C) and on the "acid" form of poly-A [20]. It was therefore suggested that the specific enhancement of stability due to diamines requires the pre-existence of a native DNA configuration [20].

A further refinement of prerequisites for the appearance of the effect induced by aliphatic amines seemed useful for clarifying their role. The relationship between the AT content and the extent of T_m enhancement suggested the advisability of examining the influence of these cations on the homopolymer of ribothymidylic acid. It is seen from the temperature melting profiles in Fig. 7 that all the alkyl diamines NH_2-$(CH_2)_n$-NH_2 tested (n=2 to 6) are more effective than Mg^{2+} in rising T_m of poly-rT at an equivalent diamine-polymer phosphate ratio of 1 : 1. Their effectivity increases with increasing chain length up to n = 5, then decreases rather

sharply. Thus, the regularity concerning diamine chain length and effectivity established for DNA by Mahler & Mehrotra [18] applies to poly-rT. These results demonstrate clearly that the substitution of ribose for deoxyribose is not essential, and point to thymine as to the secondary binding site responsible for this interaction in poly-rT as well as in DNA (see below). T_m of poly-rT is substantially enhanced by polyamines (Fig.7); however, owing to the increased number of amine groups per molecule it is difficult to compare polyamines with divalent metal ions. It should be perhaps noted that even the wide use of Mg^{2+} as a "reference" ion to compare the specific and non-specific influence of diamines may not be quite substantiated because of differences in charge distribution and in solvating power.

Further experiments at higher diamine concentration demonstrated the existence of a linear relationship of the plot of T_m *versus* logarithm concentration (Fig. 8). The results with Mg^{2+} are included for comparison and it will be observed that curves for diamines follow the same pattern, including the change in slope at an equivalent counterion-polymer phosphate ratio of 5 : 1. In the case of cadaverine, spermidine and spermine at equivalent concentrations somewhat exceeding that

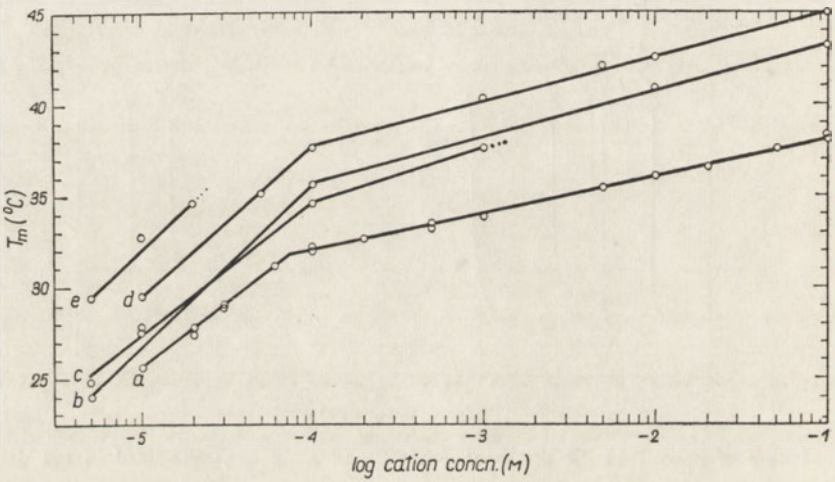

Fig. 8. Variation of T_m of poly-rT with the concentration of: *a*, Mg^{2+}; *b*, 1,2-diaminoethane; *c*, 1,6-diaminohexane; *d*, 1,4-diaminobutane; *e*, 1,5-diaminopentane. Dotted lines indicate the appearance of absorption at 340 mμ:

of the polymer, measurements at 340 mμ revealed the appearance of turbidity; the resulting precipitate was shown to be a poly-rT - polyamine complex. Precipitation of this complex is a function of polymer-polyamine affinity rather than of polyamine chain length; the poly-rT - 1,6-diaminohexane complex precipitates at a concentration exceeding 100-fold that of the polymer.

Contrary to what might have been expected, none of the diamines tested proved to be more effective than Mg^{2+} in stabilizing the poly-(A+rT) complex (Table 2). Three various ionic strengths were employed for these experiments in order to obtain T_m values within a sufficiently broad temperature range. While at low ionic

strength (Table 2, first two columns) melting of a double-stranded structure is observed, at higher ionic strength readings at 282.5 mμ reveal the rearrangement of the twin helix to a triple-stranded one [cf. 22]. In neither case were diamines more effective than Mg^{2+}.

Table 2

Variation of T_m of poly-(A+rT) with ionic strength and diamine chain length

Conditions: 6×10^{-5} M total polymer phosphate, 10^{-2} M-phosphate buffer, pH 7.8; readings at 262.5 mμ. In brackets are given T_m values for the poly-(A+U) 1 : 1 complex.

Divalent cation added	T_m (°C)		
	in 0.01 M-Na^+	in 0.04 M-Na^+	in 0.1 M-Na^+
	Divalent cation concn.		
	3×10^{-5} M	10^{-4} M	10^{-3} M
None	56.2	64.2	75.5 (56)
Mg^{2+}	57.5	67.2	81.2* (64.8*)
$NH_2(CH_2)_2NH_2$	55.5	63.5	73.3* (56.5)
$NH_2(CH_2)_4NH_2$	56.5	65.0	77.5* (58.5*)
$NH_2(CH_2)_5NH_2$	57.0	66.6	77.5* (58.1*)
$NH_2(CH_2)_6NH_2$	56.2	65.6	77.0* (57.1*)
Average Δt (°C)	3.2	3.4	4.2

* Indicates the melting of a triple-stranded structure, despite the 1:1 mixing ratio of the polymers, as indicated by readings at 282.5 mμ [22].

The fact that diamines, particularly cadaverine and putrescine (n = 4), increase the thermal stability of the alternating sequence poly-dAT [18], and of poly-rT, provided a clue that the presence of thymine in both strands could be a major factor. To examine this possibility a poly-rAT copolymer was synthesized with the help of polynucleotide phosphorylase (*A. vinelandii*) from a 1 : 1 mixture of the corresponding diphosphates. Alkaline hydrolysis of the isolated copolymer (0.3 M-KOH, 20 hr., 35°), followed by paper chromatography, elution and spectral estimation of the components demonstrated an actual base ratio thymine : adenine of 41 : 59. The melting of this random poly-rTA (41: 59) was indeed enhanced more effectively by cadaverine and putrescine than by Mg^{2+}, the corresponding T_m values being 56.5°, 54.2° and 53.5°, respectively, at 10^{-1} M-Na^+ and 10^{-3} M divalent cation. However, the copolymer exhibited a rather broad melting profile ($\Delta t = 13.7°$), and a diminished hyperchromicity (31%) as compared to the melting profiles of 1 : 1 complexes of poly-(A+rT). Examination of an alternate sequence poly-r-AT obtained with the help of DNA-dependent RNA polymerase would obviously provide a more meaningful answer.

The foregoing results concerning interactions of poly-rT with di- and polyamines suggested the advisability of examining the melting behaviour of poly-U in their presence. Poly-U was shown to exhibit some degree of ordered state near 0° and in the presence of at least 10^{-3} M-Mg^{2+} [17]. Fig. 9 reveals that all diamines tested exhibit an additional stabilizing effect which is apparently distinct from their activity as counterions. This stabilization differs from the one found for poly-rT

since it increases on reducing chain length from $C_{(6)}$ to $C_{(2)}$. Thus, the maximum stabilizing effect afforded by a given diamine is shown to be base-dependent. No immediate explanation is forthcoming for this deviation from the "normal" order

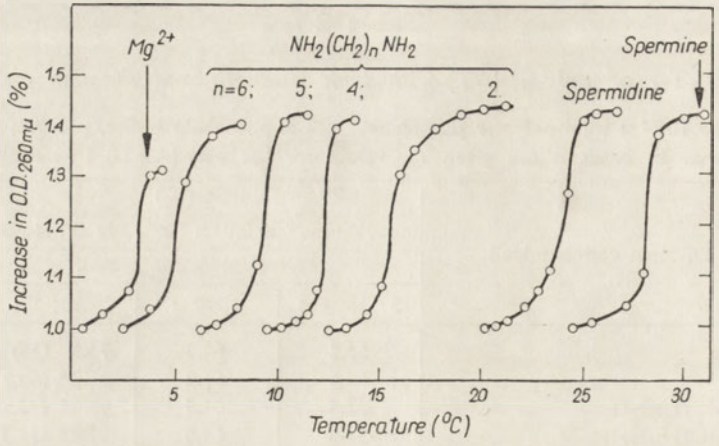

Fig. 9. Melting of poly-U in the presence of Mg^{2+} and amines as indicated in the diagram. Polymer concentration 4×10^{-5} M, concentration of divalent cations 10^{-3} M. Concentration of polyamines was equivalent to polymer phosphate.

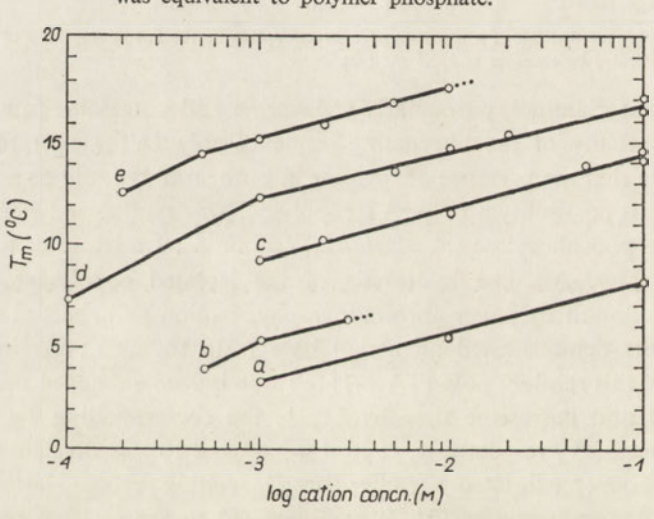

Fig. 10. Variation of T_m of poly-U with the concentration of: *a*, Mg^{2+}; *b*, 1,6-diaminohexane; *c*, 1,5-diaminopentane; *d*, 1,4-diaminobutane; *e*, 1,2-diaminoethane. Dotted lines indicate the appearance of absorption at 340 mμ.

of effectiveness of diamines in the absence of the 5-methyl group; it may be pointed out that the poly-U and poly-rT helices have apparently different dimensions, the latter being more compact.

Unlike the poly-rT case, formation of the ordered state of poiy-U was completed at divalent ion concentraton exceeding that of the polymer; it is presumably due to the low ability of poly-U to interact with itself.

Again, as for poly-rT, a linear relationship exists of the plot of T_m *versus* logarithm counterion concentration, with a similar change in slope for the counterion saturated system (Fig. 10).

It will be noted that polyamines exhibit a considerable influence on the T_m of poly-U (Fig. 9). There is a shift in helix-coil transition from several degrees above 0° at 10^{-3} M-Mg^{2+} to 25°-28° in the presence of equivalent amounts of polyamines. Thus, poly-U becomes a suitable model for investigating the impact of secondary structure at convenient temperatures. At higher spermine molarity a poly-U - spermine complex precipitates as observed earlier by Huang & Felsenfeld [13]; the concentration of spermidine may be raised to 10^{-4} M and T_m increases by a further 2.5°. The possible configuration of poly-U in the ordered state was discussed elsewhere [26].

The melting of poly-C and poly-A which are known to possess broad melting profiles at neutral pH with no indication of co-operativeness was not affected by the presence of di- and polyamines.

Table 3

Variation of T_m *of poly-(A+U) with equivalent* Mg^{2+} *and polyamine concentration*

Conditions: 6×10^{-5} M total polymer phosphate, 10^{-2} M-phosphate buffer, pH 7.8, adjusted with NaCl to final 8.2×10^{-2} M-Na^+; readings at 260 mμ.

Ratio of eqv. counterion to polymer P	T_m (°C) in the presence of		
	Mg^{2+}	spermidine^{3+}	spermine^{4+}
1 : 1	54.0	55.0	61.5
2 : 1	54.5	56.5	63.0*
3 : 1	55.2	57.9	66.5*
5 : 1	55.9	59.2*	69.1*
8 : 1	57.2*	63.5*	70.6*

* Indicates the melting of a triple-stranded structure, despite the 1:1 mixing ratio of the polymers, as indicated by readings at 280 mμ.

Felsenfeld & Huang [5] have demonstrated, using the poly-)A+U) interaction as a model, that diamines are a little less strongly bound to polymer phosphate groups than divalent metal ions since, in this instance, a slightly higher diamine concentration is necessary to bring about the formation of the complex. This is apparently reflected in the melting behaviour of poly-(A+U) in the presence of Mg^{2+} and diamines, the latter being more effective in rising T_m ([20], cf. also Table 2). Experiments on the interaction of spermidine and spermine with the homopolymers of rT and U have shown that with an increase of the number of amine groups per molecule T_m is substantially enhanced as compared to Mg^{2+} at a 1 : 1 stoicheiometry. This was also shown to be true for complexes of poly-(A+rT) and poly-(A+U). Results for the latter are presented in Table 3, from which it will be seen that the order of effectivity $\frac{Mg^{2+}}{2} < \frac{\text{spermidine}^{3+}}{3} < \frac{\text{spermine}^{4+}}{4}$ remains qualitatively the same as for the homopolymers (cf. Figs. 7 and 9).

DISCUSSION

The behaviour of poly-rT in the presence of mono- and divalent ions is indicative of a highly ordered regular structure embracing the entire polymer molecule rather than its separate regions. The helix-coil transition occurs at temperatures lower than for the complementary homopolymer pair complexes, but the main features of ordered state formation and T_m dependence on the concentration of weakly and strongly bound cations, remain qualitatively unaltered. It will be noted that in presence of high concentrations of metal ions, whether mono- or divalent, the highest attainable T_m of poly-rT is essentially the same (cf. Fig. 2 and 4) amounting to about 40°.

Much higher T_m values were obtained in the presence of di- and polyamines. The identical stoicheiometry of poly-rT ordered state formation, whether in the presence of Mg^{2+} or diamines indicates that both amine groups are involved in the neutralization of the phosphate backbone charge. However, the affinity of divalent metal ions toward the formation of poly-(I+C), poly-(A+U) [5, 20], and poly-(A+rT) is somewhat greater than that of diamines; subsequently, these structures, as well as some RNA's and single-stranded DNA [18, 20] are more effectively prevented from thermal dissociation by divalent metal ions. A quite different situation prevails for native DNA, poly-d-AT [18, 20], poly-rT and the random poly-r-TA (41 : 59), where diamines, and particularly the naturally occurring putrescine and cadaverine, exhibit a higher affinity than Mg^{2+} and other divalent metal ions. Nevertheless, the mere presence of thymine is not a sufficient prerequisite for the manifestation of the maximum effect exhibited by putrescine and cadaverine. The presence of adenine, and the substitution of ribose for deoxyribose did not prove to be essential. Summarizing the data from various investigations [18, 20], and derived from the results presented here, the following requirements appear to be essential: the potential capability of a polynucleotide to form a regular helix as manifested by a co-operative type of transition, and the presence of thymine in both strands. Only native DNA fulfills these requirements and, subsequently, only in this case, out of all natural nucleic acid molecules studied, putrescine and cadaverine were found to be more effective structure supporting agents than Mg^{2+}.

Regarding the higher amines, spermidine and spermine, they do not appear to be limited in exerting their specific influence by the above mentioned requirements. Thus, T_m values of all the U or rT containing homopolymers and complementary complexes were increased in the presence of polyamines more effectively than in the presence of divalent metal ions, referring to a stoicheiometric equivalence. Mandel [19] has shown that spermine increases T_m of sonicated, low molecular weight DNA in the same way it acts towards native DNA. Spermine was also shown to interact with turnip yellow mosaic virus RNA to make it more compact, better prevented from temperature-induced unfolding and less susceptible to digestion by ribonuclease [21]. Nevertheless, the affinity of polyamines to the homopolymers of U and rT appears to be much greater than to their complexes with poly-A as reflected by relative T_m increases (cf. data from Table 3 and Fig. 9).

The interaction of poly-rT with monoamines points to quite another regularity. These monovalent ions are weakly bound, i.e. they are apparently not bound to any specific site on the polymer (for a detailed discussion see ref. [6]), and their high concentration is required to form the ordered state. Being present in a large excess, monoamines behave toward the polymer much like some of the organic solvents known to denature DNA by breaking hydrophobic interactions [7]. As well established, an increased number of alkyl substituents on amides, ureas and alcohols enhances the denaturing effectiveness of these solvents toward DNA [10, 16, 11]. Similarly for poly-rT, NH_4^+ was shown to be most effective; any substitution, and elongation of the aliphatic chain tends to destabilize the ordered state (cf. curves *i*, NH_4; *h*, methylamine and *b*, butylamine, Fig. 6), while the substitution of a hydroxyl in the chain increases T_m (cf. curves *g*, triethylamine and *e*, triethanolamine, Fig.6). The cationic nature of monoamines provides for the formation of the ordered state and the organic moiety acts similarly as the above mentioned denaturing agents. Hence, only the aliphatic moiety of a di- or polyvalent cation, apparently strongly bound to a specific site on the polymer, is capable of conferring, under proper conditions, an increased stability.

In a sense the influence of strongly bound aliphatic amines on the ordered state of some of the investigated polynucleotides is analogous to that of a 5-methyl substituent in a pyrimidine polynucleotide. Thus, a 5-methyl group is without effect on the structure of the neutral form of poly-C [24], which by itself does not possess any sharp melting profile, but it contributes to the further enhancement of the ordered state of poly-U, the latter exhibiting a co-operative type of transition [17]. The aliphatic nature of both factors discussed, and the requirement of a pre-existing regular structure for their manifestation, suggest the involvement of hydrophobic bonding as a source of the additional stabilization.

If we take into account the above analogy, it seems justified to regard the aliphatic chain of a di- or polyamine as an inherent part of the polymer molecule, fixed, to some degree, in a certain spatial position as determined by the primary binding sites. The extent of "fixing" is presumably correlated with the number of cationic groups per molecule; this would account for the higher effectiveness of polyamines as compared to diamines. An examination of the structure of poly-rT and poly-U by direct methods, and model building would help to understand the peculiar role of thymine and the base-dependence of the effect exhibited by diamines.

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WSPÓŁDZIAŁANIE KWASU POLIRYBOZOTYMIDYLOWEGO Z JONAMI METALI I AMIN ALIFATYCZNYCH

Streszczenie

1. Badano wpływ kationów metali jedno- i dwuwartościowych na powstawanie i trwałość termiczną stanu uporządkowanego kwasu polirybozotymidylowego. Stwierdzono, że kationy jednowartościowe są słabo związane z polimerem, a kationy dwuwartościowe — silnie.

2. Podobne badania przeprowadzono nad współdziałaniem szeregu mono-, dwu- i poliamin z kwasem polirybozotymidylowym, innymi homopolirybonukleotydami oraz z komplementarnymi parami homopolimerów.

3. Określono warunki, w jakich występuje dodatkowa, specyficzna stabilizacja stanu uporządkowanego pod wpływem niektórych dwu- i poliamin, np. maksymalny stabilizujący wpływ kadaweryny i putrescyny manifestuje się w stosunku do polinukleotydu zdolnego do utworzenia uporządkowanej struktury i zawierającego tyminę w obydwu niciach. Omówiono przypuszczalny mechanizm zjawiska dodatkowej stabilizacji w stosunku do modelowych i naturalnych polinukleotydów.

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DIHYDROPYRIMIDINASE ACTIVITY IN PEA PLANTS

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1. A crude enzyme preparation that catalyses the conversion of 4,5-dihydro--orotate, 4,5-dihydrouracil, and 4,5-dihydrothymine to *N*-carbamoylaspartate, *N*-carbamoyl-β-alanine, and *N*-carbamoyl-β-aminoisobutyrate, respectively, as well as carbamoylaspartate to dihydro-orotate, has been isolated from pea plant homogenates by fractionation with ammonium sulphate. 2. The effect of pH, time, temperature, substrate and enzyme concentrations, as well as presence of divalent cations on the rate of these conversions has been described. 3. It may be concluded that enzymic activities corresponding to dihydro-orotase and dihydropyrimidinase thus far demonstrated in bacteria and animal tissues, are present in higher plants as well.

N-Carbamoyl-β-amino acids, namely *N*-carbamoyl-L-aspartate[1], *N*-carbamoyl-β--alanine, and *N*-carbamoyl-β-aminoisobutyrate, are converted to the corresponding dihydropyrimidines: dihydro-orotate, dihydrouracil and dihydrothymine in the process of the pyrimidine nucleotide biosynthesis. Enzymes responsible for these transformations, dihydro-orotase (L-4,5-dihydro-orotate amidohydrolase, EC 3.5.2.3.) and dihydropyrimidinase (4,5-dihydropyrimidine amidohydrolase, EC 3.5.2.2.) have been identified in animal tissues and bacteria. In the present investigation, the crude enzyme preparation from pea seedlings was examined for the ability to catalyse interconversion of dihydropyrimidines and carbamoyl-β-amino acids. Occurrence of dihydropyrimidinases (the term is used in the present work for the amidohydrolases of dihydropyrimidine compounds) in plant material seemed probable since higher plants were shown to utilize CA[2] [3] and CBA [2] for the pyrimidine synthesis *in vivo*.

MATERIAL AND METHODS

Preparation of enzyme. Fresh top leaves (50-100 g.) of two week old green pea plants (variety Perła Szlachetna) were homogenized with 100 - 200 ml. of

[1] Aspartic acid may be considered as β-amino acid since in this compound NH_2-group occupies β-position in regard to one of COOH-groups.

[2] The abbreviations used are: CA, *N*-carbamoyl-L-aspartate; CBA, *N*-carbamoyl-β-alanine; CBAIB, *N*-carbamoyl-β-aminoisobutyrate; DHOA, L-4,5-dihydro-orotate; DHT, 4,5-dihydrothymine; DHU, 4,5-dihydrouracil.

0.1 M-potassium phosphate buffer, pH 7.4, containing 1 mM-2-mercaptoethanol and 0.1 mM-EDTA. Homogenization and all subsequent operations were performed at 0 - 4°. The homogenate was filtered through cheesecloth and centrifuged at 6 000 *g* for 10 min. To the supernatant, solid ammonium sulphate was added with stirring to 40% saturation. After 1 hr. the precipitate was removed by centrifugation and discarded. The supernatant was made up to 65% saturation with ammonium sulphate as above and the protein fraction precipitated at 40 to 65% saturation was collected by centrifugation and stored at 0°.

Incubation. For preparation of the standard incubation mixture, portions of the protein precipitate were dissolved in 0.1 M-citrate-potassium phosphate buffer, pH 6.0, containing 1 mM-2-mercaptoethanol and 0.1 mM-EDTA. To 0.2 ml. portions of the solution, containing about 1 mg. of protein, 1 μmole of the appropriate substrate dissolved in 0.02 ml. of the same buffer was added and the mixture incubated at 37°. The reaction was terminated after 2 hr. by the addition of 5.0 ml. of 0.3 N-perchloric acid.

Analytical methods. The carbamoyl-β-amino acids were estimated colorimetrically by the method of Reifer & Toczko [15]. Separate standard curves were prepared for CA, CBA, and CBAIB determinations. The individual carbamoyl-β-amino acids were identified by paper chromatography on Whatman no. 1 paper in propan--1-ol - water - HCl (30 : 10 : 1, by vol.) and butan-1-ol - water - acetic acid (2 : 1 : 1, by vol.) solvent systems. The carbamoyl spots were detected by the colour test described by Fink *et al.* [7].

The dihydropyrimidines were determined spectrophotometrically at 230 mμ, according to the method of Janion & Shugar [8], after alkalization of the deproteinized incubation mixtures. Identification of DHOA was based on measurements of its ultraviolet absorption spectra using the spectrophotometer SF-4 (U.S.S.R.), on its characteristic alkali-lability as well as on the R_F values. Conditions for the paper chromatography were the same as in the case of carbamoylamino acids. Detection of DHOA on paper chromatograms was carried out according to the procedure of Fink *et al.* [7].

Protein was determined by the colorimetric method of Lowry *et al.* [11].

Chemicals. L-4,5-Dihydro-orotate, 4,5-dihydrouracil, 4,5-dihydrothymine and β-alanine were purchased from Calbiochem. (Los Angeles, U.S.A.); 2-mercaptoethanol was obtained from Koch-Light Co. (Colnbrook, England); *N*-carbamoyl--L-aspartate was a gift from Prof. Dr. H. J. Sallach (Madison, U.S.A.); *N*-carbamoyl-β-alanine was synthesized from β-alanine and potassium cyanate according to the method of Nyc & Mitchell [14]; other reagents were of analytical grade, obtained from Fabryka Odczynników Chemicznych, Gliwice, Poland.

RESULTS

Three dihydropyrimidine compounds, DHOA, DHU, and DHT, were used as substrates to test the catalytic activity of the crude enzyme preparation isolated from pea seedlings (Table 1). The decrease in the amount of the dihydropyrimidines

Table 1

Conversion of the dihydropyrimidines to carbamoyl compounds by the enzyme preparation from pea seedlings

Incubation was carried out as described in the Material and Methods section, except that 0.1 M-potassium phosphate buffer, pH 8, was used. Data for zero time incubation are not included since no changes were observed.

Substrate	Incubation mixture	Substrate metabolized (μmole)	Carbamoyl compounds synthesized (μmole)
L-4,5-Dihydro-orotate	Complete system	0.30	0.37
	Substrate omitted	0.00	0.04
	Enzyme omitted	0.00	0.06
4,5-Dihydrouracil	Complete system	0.37	0.40
	Substrate omitted	0.00	0.04
	Enzyme omitted	0.00	0.00
4,5-Dihydrothymine	Complete system	0.25	0.30
	Substrate omitted	0.00	0.04
	Enzyme omitted	0.00	0.00

Table 2

Conversion of N-*carbamoyl*-L-*aspartate to* L-4,5-*dihydro-orotate*

Standard conditions, as described in Methods; pH 6; concn. of divalent cations 10^{-4} M

Incubation mixture	Substrate metabolized (μmole)	Product formed (μmole)
Complete system	0.36	0.27
Substrate omitted	0.04	0.00
Enzyme omitted	0.00	0.00
EDTA omitted	0.34	0.22
2-Mercaptoethanol omitted	0.35	0.22
EDTA and 2-mercaptoethanol omitted	0.24	0.20
EDTA omitted, Co^{2+} added	—	0.20
EDTA omitted, Mg^{2+} added	—	0.21
EDTA omitted, Zn^{2+} added	—	0.20

was accompanied by a corresponding increase in the amount of carbamoyl compounds. These were tentatively identified as CA, CBA and CBAIB when DHOA, DHU and DHT, respectively, were used as substrates. The synthesis of each of them was of the same order and roughly equimolar in respect to the decrease of the corresponding dihydropyrimidines. Three types of control experiments: without enzyme, without substrate and zero time incubation, adequately excluded any possibility of non-enzymic reactions.

One of the carbamoylamino acids, viz. CA, was employed to ascertain whether the opposite direction of the investigated reactions may also be catalysed by the same enzyme preparation (Table 2). It was found that the cyclization of CA to DHOA is of the same order as the reaction in the opposite direction. Omission of both EDTA and 2-mercaptoethanol from the system decreased the enzyme activity.

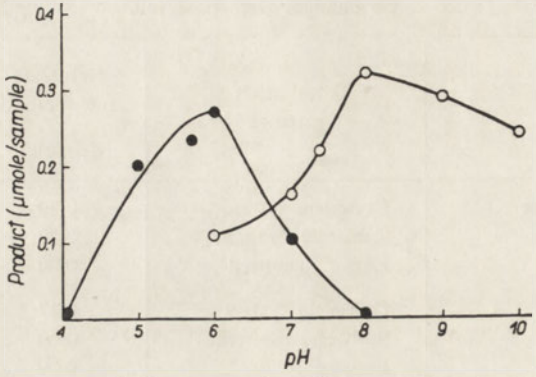

Fig. 1. Effect of pH on enzymic interconversion dihydro-orotate ⇌ carbamoylaspartate: (○), carbamoylaspartate formed; (●), dihydro-orotate formed. Assay conditions as described in the Material and Methods section. Buffer solutions: 0.1 M-potassium phosphate buffer, pH 6 - 10; 0.1 M-citrate-phosphate buffer, pH 4 - 8.

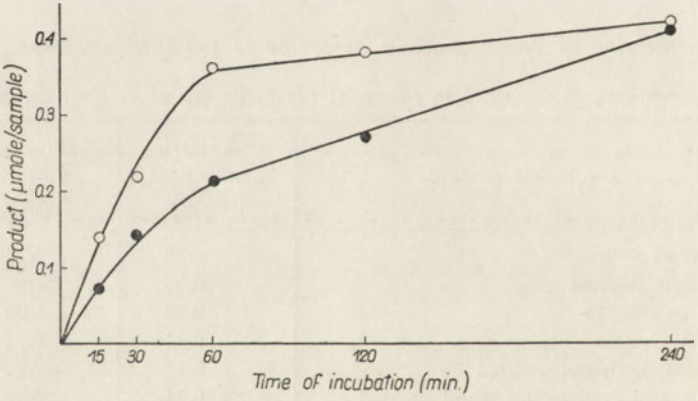

Fig. 2. Effect of time on dihydro-orotase activity: (○), conversion of dihydro-orotate to carbamoylaspartate; (●), conversion of carbamoylaspartate to dihydro-orotate. Assay conditions as described in the Material and Methods section, except that for expt. (○), pH was 8.0.

Divalent cations, that were found to be essential for bacterial dihydro-orotase activity [16], had no stimulatory effect on its plant counterpart. Two additional observations were made that seem to exclude the possibility of participation of divalent cations or any other soluble cofactors in the observed reactions. First, the enzyme precipitated by ammonium sulphate retained its high activity (tenfold purification being obtained); secondly, dialysis had no effect on the catalytic activity of the preparation.

Different pH profiles were observed for both directions of the CA⇄DHOA interconversion (Fig. 1). The optimum pH for the conversion of CA to DHOA was at pH 6, whereas for the opposite direction it was pH 8. Time-course curves were also different for the two directions (Fig. 2). When DHOA was used as substrate the progress curve was close to linear for approximately 60 min., and equilibrium was attained when about 36% of the substrate was converted to CA. Cyclization of CA to DHOA showed linear response with time up to 30 min. and then the rate of the reaction decreased considerably.

Some further informations concerning the activity of the enzyme preparation toward CA are shown in Fig. 3. The rate of the reaction was proportional to enzyme

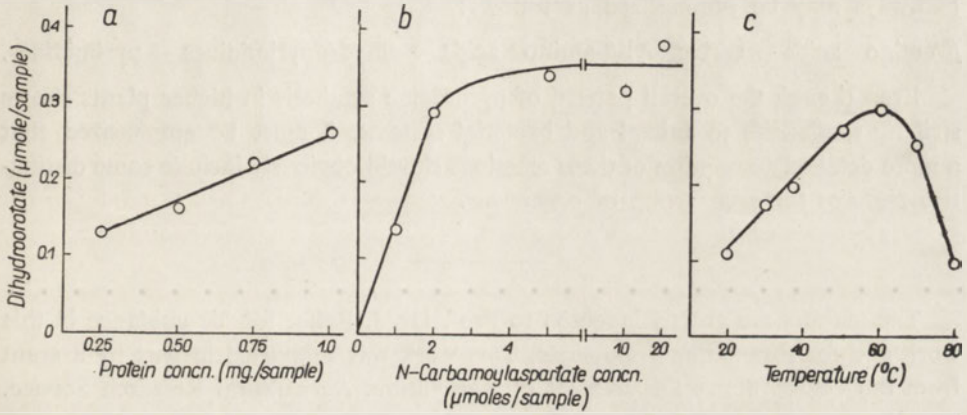

Fig. 3. Effect of (*a*), enzyme concentration; (*b*), substrate concentration, and (*c*), temperature, on the rate of dihydro-orotate formation. Assay conditions as described in the Material and Methods section, except that in Fig. 3c the time of incubation was 1 hr.

concentration up to 1 mg. of protein per sample, the highest concentration tested (Fig. 3a). Fig. 3b shows the effect of substrate concentration on activity. The temperature optimum was about 60° with a sharp decline of activity at 80° (Fig. 3c).

DISCUSSION

The conversion of DHOA, DHU and DHT to CA, CBA and CBAIB, respectively, as well as the cyclization of CA to DHOA were shown to be catalysed by a crude enzyme preparation from pea plants. Two different enzymes able to catalyse these reactions have been found in organisms other than higher plants. Dihydro-orotase, responsible for the reversible conversion of CA to DHOA, has been demonstrated in *Zymobacterium oroticum* [10, 16], *E. coli* [22, 20], rat liver [6, 19], human erythrocytes [17], and Novikoff ascites tumor cells [1]. Dihydropyrimidinase, active towards DHU as well as DHT, has been found in *Clostridium uracilicum* [4], rat [5] and ox [21] livers. It is difficult to decide at present how many enzymes participate in the above reactions detected in plant material. Indeed, they could be catalysed as well by one enzyme as by the three specific enzymes. Moreover, a possibility that both directions of the CA⇄DHOA interconversion were catalysed by two separate enzymes, cannot be excluded. Such a possibility is not unlikely as two different

pH profiles were observed for the cyclization of CA to DHOA and for the conversion of DHOA to CA. Comparison of the time-course curves may also lead to a similar conclusion. Further investigations and especially further purification of the enzyme preparations are necessary for identification of the enzymes involved.

However, it may be already concluded that enzymic activities of high order corresponding to dihydro-orotase and dihydropyrimidinase were detected in pea plants. Our present findings, together with data on plant aspartate transcarbamoylase [13, 12, 18] and DHOA dehydrogenase [9], suggest conclusively that, in general, the pathways of pyrimidine biosynthesis proposed for animal tissues and micro-organisms are operating in higher plants as well. In a most general form these pathways may be summarized as follows:

β-amino acids $\rightarrow$ carbamoyl-β-amino acids $\rightarrow$ dihydropyrimidines $\rightarrow$ pyrimidines.

Even though the overall pattern of pyrimidine synthesis in higher plants shows striking similarities to animal and bacterial systems, it must be emphasized that a more detailed description of these reactions should obviously include some distinctive features for each group of organisms.

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DWUHYDROPIRYMIDYNAZOWA AKTYWNOŚĆ W SIEWKACH GROCHU

Streszczenie

1. Homogenaty siewek grochu frakcjonowano siarczanem amonowym. Osad białkowy uzyskany przy 45 - 60% nasycenia posiadał zdolność katalizowania następujących przemian: L-4,5-dwuhydroorotanu do *N*-karbamoilo-L-asparaginianu, 4,5-dwuhydrouracylu do *N*-karbamoilo-β-alaniny, 4,5-dwuhydrotyminy do *N*-karbamoilo-β-aminoizomaślanu, oraz *N*-karbamoilo-L-asparaginianu do L-4,5-dwuhydroorotanu.

2. Przebadano wpływ pH, temperatury i czasu inkubacji, stężenia substratu i enzymu, oraz obecności kationów dwuwartościowych na intensywność przemian.

3. Uzyskane wyniki dowodzą, że dwuhydroorotaza i dwuhydropirymidynaza, znane dotychczas z badań przeprowadzanych na materiale bakteryjnym i zwierzęcym, występują także i w roślinach wyższych.

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PROPERTIES OF *ESCHERICHIA COLI* RIBOSOMES OBTAINED BY PRECIPITATION AT LOW pH VALUES*

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1. Most of the ribosomes of *E. coli* precipitated between pH 5.8 and 5.2. At pH 5.0 virtually no ribosomes remained in the supernatant. 2. Some soluble proteins precipitated together with ribosomes at pH 5.2 and they were separated by sucrose gradient centrifugation. 3. The ribosomes obtained by precipitation from acidic solutions dissociated into 50s and 30s sub-units when the concentration of Mg^{2+} was lowered to 0.1 mM, and reassociated to give 70s particles when 10 mM concentration of Mg^{2+} was restored. 4. Ribosomes precipitated between pH 5.8 and 5.2 were active in the poly-U directed incorporation of phenylalanine, the activity being the same as that of ribosomes obtained by differential centrifugation.

The role of ribosomes as the site of protein biosynthesis is now generally accepted; however, despite extensive investigation, many properties and details of structure of these ribonucleoprotein complexes are still obscure.

Until now, differential centrifugation was used as the only method of isolation and purification of ribosomes from various cells [9]. In 1965 Arnstein *et al.* [1] have reported that reticulocyte ribosomes could be reversibly precipitated at pH 6, and it appeared that this procedure did not alter the physico-chemical or biological properties of the ribosomes. Our observations on liver extracts [11] and later on *E. coli* cell-free preparations suggested that the capability of reversible precipitation at low pH values is not unique for reticulocyte ribosomes but is a general property of ribosomes from different types of cells. Therefore it seemed interesting to study the properties of ribosomes obtained from *E. coli* extracts by precipitation at low pH and compare them with the properties of ribosomes isolated by classical methods of differential centrifugation. We have selected *E. coli* because their ribosomes have been most extensively studied and the results obtained by the new isolation procedure could be compared with the data obtained by other methods.

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* The preliminary report of this work has been presented at the Third FEBS Meeting, Abstr. G 38, Warsaw, 1966.

MATERIALS AND METHODS

Reagents. L-[U-^{14}C]Phenylalanine (10 mc per 1 m-mole) was a product of Amersham Radiochemical Centre, Bucks, England. ATP and potassium phosphoenolpyruvate were obtained from Boehringer und Soehne GmbH, Mannheim, Germany; GTP and ATP : pyruvate phosphotransferase from Sigma Chemical Co., St. Louis, Miss. U.S.A.

s-RNA was prepared from *E. coli* cells by the method of Warner & Speyer (cf. Szer & Ochoa [13]). Polyuridylic acid (poly-U) prepared by the method of Grunberg-Manago *et al.* [3] was a kind gift of Dr. W. Szer of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw.

The standard buffer of Nirenberg & Matthaei [8] containing 0.01 M-tris-HCl pH 7.8, 0.06 M-KCl or NH_4Cl [12], and 0.014 M-magnesium acetate was used throughout.

The preparation of cell-free extracts. Escherichia coli B was cultivated on the medium described by Littauer & Kornberg [5] at 30° with vigorous aeration. The cells were harvested in the early logarithmic phase by centrifugation in the cold, washed once with the standard buffer of Nirenberg & Matthaei [8] and stored at -20° until needed. After thawing the cells were suspended in 1 - 2 volumes of standard buffer and disrupted in the Eaton's press [2] at a pressure of 6 - 8 tonns. The supernatant at 30 000 *g* (S-30 fraction) was prepared from this homogenate as described by Nirenberg & Matthaei [8]. It was used immediately or stored at -20° not longer than a few weeks.

The preparation of ribosomes by differential centrifugation. The S-30 fraction was centrifuged at 105 000 *g* for 2 hr. at 0 - 3°. The ribosomal pellet was resuspended in the standard buffer by gentle homogenization and the insoluble material removed by centrifugation at 20 000 *g*. The supernatant was again centrifuged for 2 hr. at 105 000 *g*. The final pellet was suspended in the standard buffer and the insoluble material removed at 20 000 *g*. The ribosomes were stored at -20°. The supernatant after the first centrifugation at 105 000 *g* was frozen and used as the source of the soluble enzymes in the amino acid incorporation experiments.

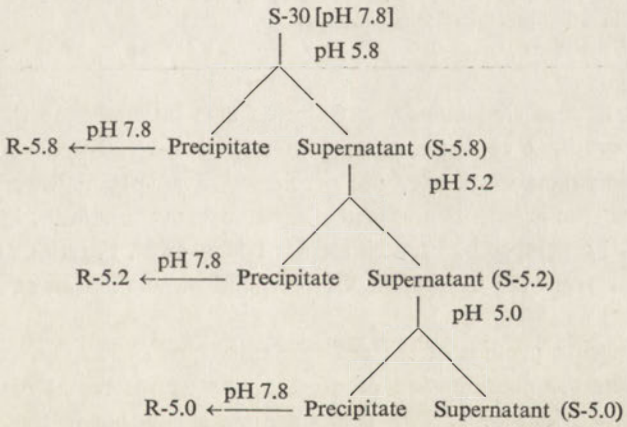
Amino acid incorporation by the cell-free system. Poly-U dependent incorporation of phenylalanine was determined essentially by the method described by Nirenberg & Matthaei [8]. The incubation mixture contained in 0.25 ml.: 25 μmoles tris-HCl, pH 7.8; 3.5 μmoles magnesium acetate; 13 μmoles KCl or NH_4Cl [12]; 1.5 μmoles 2-mercaptoethanol; 0.25 μmole ATP; 0.1 μmole GTP; 5 μg. ATP : pyruvate phosphotransferase; 1.3 μmoles phosphoenolpyruvate; 0.75 mg. s-RNA; 20 μg. poly-U; 0.05 μmole [^{14}C]phenylalanine (0.5 μc); 1 - 2 mg. ribosomes; and 105 000 *g* supernatant (1 - 2 mg. protein). The incubation was carried out for 1 hr. at 37°. The reaction was stopped by the addition of 10% trichloroacetic acid (TCA). The precipitate was washed with hot 5% TCA, twice with cold 5% TCA, ethanol and ether. The radioactivity in the precipitated protein was determined in the liquid scintillator containing 11 ml. of 0.3% *p*-diphenyloxazole in toluene, 3.5 ml. absolute ethanol and 0.3 ml. of protein solution in conc. formic acid. The counting efficiency was 50%.

Density gradient centrifugation. Sucrose solutions were prepared in the standard buffer with Mg^{2+} ion concentration of 14 mM or 0.1 mM as indicated in the text. 4 ml. linear gradients of sucrose 5% to 20% and 15% to 30% were prepared according to Martin & Ames [7]. 30 ml. linear gradients were prepared according to Arnstein *et al.* [1]. After centrifugation fractions were collected by drop counting from the bottom of the tubes. Sedimentation coefficients were approximated as described by Martin & Ames [7].

Analytical methods. Protein was determined by the method of Lowry *et al.* [6]. RNA was determined by the method of Schneider [10] or spectrophotometrically at 260 mμ using the extinction coefficient $E_{1\,cm.}^{1\%} = 220$. The pH was measured potentiometrically with glass electrode and a pH-meter with lamp amplifier.

RESULTS AND DISCUSSION

The fractionation of cell-free extracts at low pH and all other operations with the fractions were performed at 4° unless otherwise indicated. Fraction S-30, 20 to 50 ml., obtained from 10 to 15 g. (wet wt.) of logarithmically growing cells was brought slowly to pH 5.8 with 0.1-0.5 N-acetic acid. The precipitate was centrifuged off at 11 000 *g*. The supernatant (S-5.8) was brought slowly to pH 5.2 with acetic acid and the bulky precipitate which formed was collected by centrifugation at 11 000 *g*. The supernatant (S-5.2) was acidified further to pH 5.0 and the precipitate collected by centrifugation. The supernatant was called S-5.0.



Scheme 1. The fractionation pattern of *E. coli* cell-free extract at acidic pH values.

The successive precipitates were immediately suspended in the standard buffer and if necessary the pH was adjusted to 7.8 with concentrated tris buffer, pH 8.5. Any insoluble material was removed by centrifugation at 11 000 *g* and the precipitation repeated twice more. The final preparations were dissolved in the standard buffer and after the removal of insoluble material quickly frozen and stored at -20° until needed. The preparations obtained by precipitation at pH 5.8, between pH 5.8 and 5.2, and between 5.2 and 5.0 were called R-5.8, R-5.2 and R-5.0, respectively. The above fractionation pattern is presented in Scheme 1.

The precipitation of ribonucleoprotein from the cell-free extract was followed by the determination of total ribonucleic acid in the supernatants after each precipitation. The results presented in Table 1 indicate that about 77% of the total RNA present in the initial cell-free extract of *E. coli* was precipitated between pH 5.8 and 5.2. It is a well established fact that about 80% of the total RNA in bacterial cell represents the ribosomal RNA. The above results suggest that most of the ribosomes present in the cell-free extract have precipitated between the pH 5.8 and 5.2.

Table 1

The precipitation of RNA from the cell-free extracts of E. coli *at different* pH *values*

The details of the fractionation procedures are described in the text. Portions of each supernatant were pipetted into cold 5% trichloroacetic acid and used for the determination of RNA by the method of Schneider [10].

Fraction	Total RNA in the fraction (mg.)	The RNA precipitated	
		(mg.)	(%)
Supernatant after centrifugation at 30 000 *g* (S-30)	54	—	—
Supernatant after precipitation at pH 5.8 (S-5.8)	51.7	2.3	4.2
Supernatant after precipitation between pH 5.8 and 5.2 (S-5.2)	10.0	41.7	77.2
Supernatant after precipitation between pH 5.2 and 5.0 (S-5.0)	6.4	3.6	6.6

The precipitation of ribosomes was followed also by analysing the fractions by sucrose density-gradient centrifugation. One ml. of the cell-free extract (fraction S-30) and of the supernatant after the precipitation at pH 5.0 were applied on top of 30 ml. sucrose gradients and centirfuged at 25 000 rev./min. in a fixed angle rotor of the MSE superspeed-25 centrifuge for 1.5 hr. at 0°. Fractions were collected by drop counting from the bottom of the tube and the extinction at 260 mμ determined.

The sedimentation profiles of the cell-free extract of *E. coli* and of the supernatant fraction after the precipitation of most of the ribosomes at pH 5.0 (fraction S-5.0) are presented in Fig. 1. Two peaks are clearly visible in the sedimentation pattern of the S-30 fraction (Fig. 1a); one of them corresponds to single ribosomes, another, more heterogeneous and sedimenting at a much higher rate, corresponds to the position of polyribosomes. The supernatant after the precipitation of ribosomes at pH 5.0 analysed in the same way by gradient centrifugation, revealed only a trace of material in the position of single ribosomes (Fig. 1b), which indicates that at pH 5.0 ribosomes have precipitated almost quantitatively.

The ribosomes precipitated between pH 5.8 and 5.2 were also analysed by sucrose density-gradient centrifugation. About 0.15 mg. of the R-5.2 preparation in 0.1 ml. standard buffer was applied on top of a 4 ml. linear sucrose gradient and

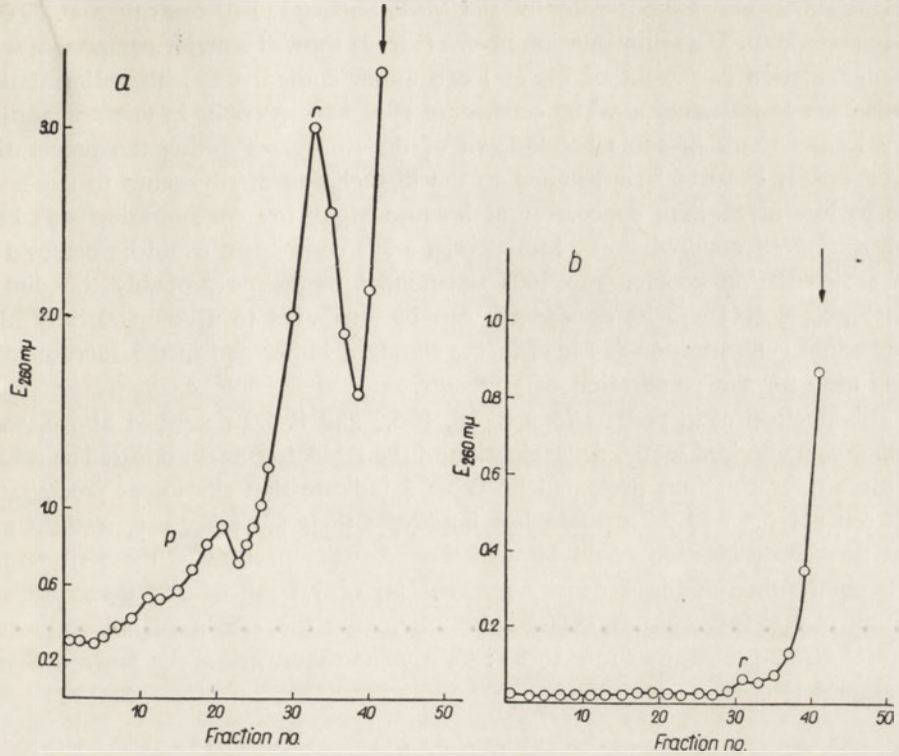


Fig. 1. The sedimentation profile of the cell-free extract of *E. coli* and the supernatant after precipitation of ribosomes at pH 5.0 (*a*), One ml. of the 30 000 *g* supernatant of disrupted *E. coli* cells was applied on top of a 30 ml. linear sucrose gradient (15% to 30%) and centrifuged at 25 000 rev./min. in an angle rotor of the MSE superspeed-25 centrifuge at 0° for 1.5 hr.; 0.8 ml. fractions were collected from the bottom of the tube and extinction at 260 mμ determined. (*b*), Supernatant after the precipitation of ribosomes at pH 5.0 was brought to pH 7.8. One ml. of this supernatant was applied on top of a sucrose gradient and centrifuged as described in (*a*).(*r*), Ribosomes; (*p*), polyribosomes.

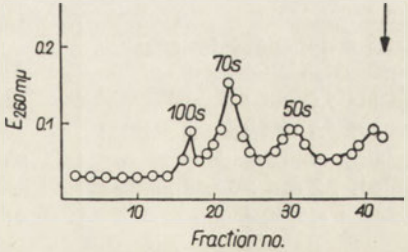


Fig. 2. The sedimentation profile of ribosomes obtained by precipitation between pH 5.8 and 5.2. The precipitate was dissolved in the standard buffer and insoluble material removed at 30 000 *g*; 0.1 ml. of the clarified solution, containing about 1.5 mg. of ribosomes was applied on top of a 4 ml. linear sucrose gradient and centrifuged in a swing-out rotor of the MSE superspeed-40 centrifuge at 39 000 rev./min. and 0° for 125 min.; 0.1 ml. fractions were collected and extinction at 260 mμ was determined.

centrifuged in a swing-out rotor of the MSE Superspeed-40 centrifuge at 39 000 rev./min. and 0°. The sedimentation profile (Fig. 2) showed a major component with a sedimentation coefficient of 70s and two minor components with sedimentation coefficients of 100s and 50s. The occurrence of the 50s particles in this preparation may be the result of some break-down of the ribosomes during the preparative procedure. It can not be attributed to the dissociation of ribosomes to sub-units due to low magnesium concentration because Mg^{2+} ion concentration was kept high during all manipulations. Moreover, no 30s component could be detected in the sedimentation profile. The 100s component represents probably the dimers of ribosomes [14] and its appearance can be attributed to a comparatively high magnesium concentration (14 mM) in the standard buffer and in the sucrose solutions used for the preparation of gradients.

Ribonucleoprotein preparations R-5.8, R-5.2 and R-5.0 described above, were further characterized by the determination of the RNA to protein ratios. The results of these determinations presented in Table 2 indicate that ribosomes precipitated between pH 5.8 and 5.2 contain less impurities than the other preparations and that further purification could be achieved after the passage of these ribosomes through the discontinuous density gradient [4] of 7.5 ml. of 0.25 M-sucrose and 7.5 ml. of 0.15 M-sucrose at 78 000 *g* for 3 hr. After this step the RNA to protein ratio of R-5.2 preparation rose to 60 : 40, a value characteristic for purified *E. coli* ribosomes [9].

Table 2

The RNA to protein ratio in ribosomal preparations obtained by precipitation from acidic solutions

Portions of the ribosomal preparations obtained as described in the text were pipetted into cold 5% trichloroacetic acid and the RNA was determined by the method of Schneider [10] and protein by the method of Lowry *et* al. [6]. The results are expressed as percentages of the sum.

Ribosomal preparation	RNA (%)	Protein (%)
Precipitated at pH 5.8	42.4	57.6
Precipitated at pH 5.8 and purified by preparative gradient centrifugation	50.6	49.4
Precipitated between pH 5.8 and 5.2	54.5	45.5
Precipitated between pH 5.8 and 5.2 and purified by preparative gradient centrifugation	60	40
Precipitated between pH 5.2 and 5.0 and purified by preparative gradient centrifugation	40	60

The dissociation of ribosomes from R-5.2 preparation at low magnesium concentration was obtained by dialysing the preparation for 24 hr. against the standard buffer containing 0.1 mM-Mg^{2+}. One ml. of the dialysed R-5.2 containing 1 mg. of protein was applied on top of the 30 ml. linear sucrose gradient (5% to 20%) in the standard buffer with low magnesium concentration (0.1 mM) and centrifu-

ged in an angle rotor of the MSE centrifuge at 25 000 rev./min. for 2.5 hr. The sedimentation profile of this preparation (Fig. 3) shows two distinct components. The sedimentation coefficients of these components determined by sucrose density-gradient centrifugation in a separate experiment were approximately 50s for the faster-moving component and 30s for the slower one.

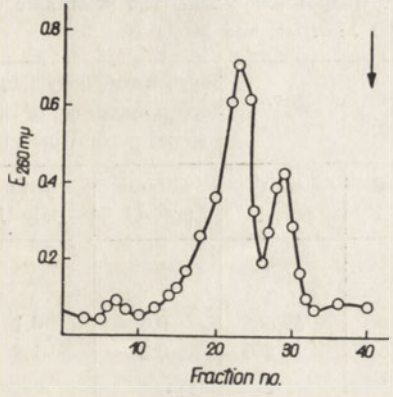


Fig. 3

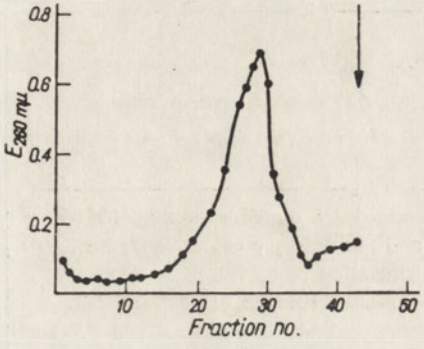


Fig. 4

Fig. 3. Dissociation of ribosomes obtained by precipitation from acidic solutions at low Mg^{2+} concentration. Ribosomes precipitated between pH 5.8 and 5.2 were dialysed against standard buffer with 0.1 mM-Mg^{2+}. One ml. of the dialysed solution containing about 1 mg. of protein was applied on top of a 30 ml. linear sucrose gradient (5% to 20%) in the standard buffer with 0.1 mM-Mg^{2+} and centrifuged in an angle rotor at 25 000 rev./min. at 0° for 2.5 hr.; 0.8 ml. fractions were collected and extinction at 260 mμ determined.

Fig. 4. The reassociation of ribosomal sub-units after the restoration of high magnesium concentration. The ribosomes obtained by precipitation between pH 5.8 and 5.2 and dissociated into sub-units as in Fig. 3 were dialysed against standard buffer with 14 mM-Mg^{2+} and analysed by sucrose gradient centrifugation as in Fig. 1 except that the centrifugation time was 2.5 hr.

After the restoration of high Mg^{2+} ion concentration by dialysing the dissociated R-5.2 preparation against the standard buffer with 14 mM-Mg^{2+}, the reconstitution of the 70s particles from the above sub-units could be observed. The sedimentation pattern of the reconstituted ribosomes is presented in Fig. 4.

The only biological function of ribosomes which is now well established is their function in polypeptide synthesis directed by specific messenger RNA. The poly-U directed incorporation of [^{14}C]phenylalanine was determined as a test of biological activity in the ribosomes obtained by precipitation from acidic solutions. The results of these determinations (Table 3) showed that ribosomes precipitated between pH 5.8 and 5.2 incorporated phenylalanine at a much higher rate than the preparation obtained at pH 5.8. After the purification by preparative gradient centrifugation the poly-U dependent incorporation of phenylalanine increased in the R-5.2 preparation more than twice reaching the same level as for purified ribosomes obtained by a classical method of differential centrifugation.

Table 3

Activity of ribosomal preparations in a poly-U dependent [^{14}C]*phenylalanine incorporation system*

The composition of the incubation mixture is described under Materials and Methods. The mixture (0.25 ml.) without or with the addition of 20 μg. of poly-U was incubated at 30° for 60 min. The reaction was stopped by the addition of 5 ml. of 10% trichloroacetic acid. The precipitate was washed and counted as described under Materials and Methods.

Ribosomal preparation	Counts/min./mg. ribosomal protein		mμmoles of phenylalanine incorporated/mg. of ribosomal protein in 1 hr.	
	without poly-U	with poly-U	without poly-U	with poly-U
Obtained by centrifugation at 105 000 *g* and purified by preparative gradient centrifugation	3018	50 666	0.3	4.1
Precipitated between pH 5.8 and 5.2	2976	22 660	0.2	1.8
Precipitated between pH 5.8 and 5.2 and purified by preparative gradient centrifugation	3622	42 752	0.25	3.7
Precipitated at pH 5.8 and purified by preparative gradient centrifugation	—	6 860	—	0.57

It is evident from the results of the above experiments that native ribosomes can be obtained from the cell-free bacterial extracts by precipitation from acidic solution. It seems that the precipitation does not alter the structure of the 70s particle and does not diminish the ability of ribosomes to accept synthetic messengers such as poly-U, and to incorporate amino acids in a messenger-dependent cell-free system.

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WŁASNOŚCI RYBOSOMÓW Z *ESCHERICHIA COLI* OTRZYMANYCH PRZEZ WYTRĄCANIE W NISKIM pH

Streszczenie

1. Większość rybosomów wytrąca się z bezkomórkowych ekstraktów *E. coli* pomiędzy pH 5.8 i 5.2. Przy pH 5.0 w supernatancie praktycznie nie było już rybosomów.

2. Część rozpuszczalnych białek wytrąca się razem z rybosomami przy pH 5.2. Białka te były oddzielone od rybosomów przez wirowanie w gradiencie sacharozy.

3. Rybosomy otrzymane przez wytrącanie w niskim pH dysocjują na podjednostki 50s i 30s przy obniżeniu stężenia Mg^{2+} do 0.1 mM i reasocjują tworząc cząstki 70s przy ponownym zwiększeniu stężenia Mg^{2+} do 10 mM.

4. Rybosomy wytrącone pomiędzy pH 5.8 i 5.2 prowadzą aktywne włączanie fenyloalaniny stymulowane przez dodatek poli-U. Aktywność włączania fenyloalaniny przez rybosomy otrzymane drogą wytrącania w niskim pH była taka sama jak aktywność rybosomów otrzymanych przez różnicowe wirowanie.

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RECENZJE KSIĄŻEK

A. D. McLaren and D. Shugar: PHOTOCHEMISTRY OF PROTEINS AND NUCLEIC ACIDS. Pergamon Press, London 1964; str. 449, cena £ 5.

Literatura dotycząca struktury oraz funkcji kwasów nukleinowych i białek została ostatnio wzbogacona książką na temat fotochemii tych związków. Dzieło to jest wynikiem współpracy dwóch wybitnych specjalistów z dziedziny fotobiologii, A. D. McLarena z Kalifornijskiego Uniwersytetu w Berkeley oraz D. Shugara z Instytutu Biochemii i Biofizyki PAN w Warszawie. Książka ta jest przeglądem dotychczasowych prac nad wpływem promieniowania nadfioletowego i widzialnego na białka i kwasy nukleinowe oraz na produkty ich degradacji. Jest ona przeznaczona głównie dla specjalistów, niemniej jednak zawiera wiele podstawowych wiadomości z dziedziny fotobiologii. Z tego względu można by ją podzielić na dwie części. Część pierwsza zaznajamia czytelnika z prawami rządzącymi fotochemią, czynnikami decydującymi o reakcji fotochemicznej, absorpcji i luminescencji nukleoproteidów oraz ich składowych części. Do tej części książki można by zaliczyć również rozdziały na temat działania promieni nadfioletowych na wiązania peptydowe, aminokwasy i pochodne aminokwasowe, jak również wpływu tego promieniowania na strukturę białka.

Druga część książki jest bardziej specjalistyczna. Obejmuje ona fotochemiczną i fotouczuleniową inaktywację enzymów, fotochemię pochodnych purynowych i pirymidynowych, wpływ promieniowania na własności transformujące DNA, inaktywację wirusów i bakteriofagów, problemy fotoreaktywacji i niektóre inne wybrane zagadnienia fotobiologii. W wielu miejscach Autorzy przedstawiają własne hipotezy, oparte głównie na wynikach swoich i współpracowników, które są stymulujące i mogą doczekać się w przyszłości potwierdzenia. Bardzo cennym uzupełnieniem książki jest dodatek poświęcony technice fotochemicznej, umożliwiający zainteresowanym wybór źródła promieniowania, odpowiedniego filtru i właściwego pomiaru intensywności światła. Bibliografia jest imponująca; obejmuje 36 stron i zawiera najnowsze publikacje dostępne przed wydrukowaniem książki.

Photochemistry of Proteins and Nucleic Acids jest pozycją która z pewnością zostanie przyjęta z wielkim uznaniem przez biochemików zajmujących się związkami wysokocząsteczkowymi.

Przemysław Szafrański

REGENERATION AND WOUND HEALING (G. Szánto ed.) Symposia Biologica Hungarica. Akadémiai Kiadó, Budapest 1964; str. 148.

Tom ten stanowi sprawozdanie z Sympozjum, które odbyło się w Budapeszcie w listopadzie 1960 r. Przedstawiono w nim 9 referatów na temat badań nad regeneracją i gojeniem się ran. Badania te były prowadzone w różnych ośrodkach naukowych i najczęściej dotyczą bardziej szczegółowych tematów.

B. P. Tokin z Leningradu proponuje rozróżnienie między zjawiskami regeneracji, t.j. odtwarzaniem utraconej części organizmu, a somatyczną embriogenezą, czyli rozwojem całego organizmu z komórek somatycznych. J. Niweliński z Krakowa przedstawił wyniki badań nad wewnątrzkomórkowymi enzymami w regenerujących kończynach traszki, R. Tsanev z Sofii omówił wpływ urazu

na nukleoproteidy protoplazmy, a B. Keller i J. Sugar w dwu referatach porównali dynamikę regeneracji naskórka w przebiegu gojenia ran ze wzrostem raka skóry. Wyniki badań histochemicznych i biochemicznych na tkance otrzymanej z okolicy wrzodu podudzia przedstawili I. Krompecher i L. Szodoray, a G. Szántó, O. Székely i S. Szonyi opisali szybsze gojenie się ran w okolicy głowowej w porównaniu z okolicą ogonową. G. Peer, J. Juhasz, J. Balint opracowali metodę wywoływania doświadczalnej nekrozy aseptycznej kości, a B. Faludi opisał wpływ kwasu fenoksyoctowego na wzrost i oporność tkanek roślinnych.

Tom ten może zainteresować czytelnika zajmującego się problemami regeneracji i gojenia się ran. Można mieć jednak żal do Wydawcy o to, że referaty sympozjalne ukazały się drukiem dopiero w cztery lata po odbyciu się Sympozjum.

Jerzy Kawiak

MAMMALIAN CYTOGENETICS AND RELATED PROBLEMS IN RADIOBIOLOGY (C. Pavan, C. Chagas, O. Fcota-Pessoa, L. R. Caldas, eds.) Pergamon Press, Oxford-New York-Paris 1964; str. 427; cena £ 5.

Książka zawiera materiały sympozjum poświęconego cytogenetyce ssaków i pokrewnym zagadnieniom w radiobiologii, które odbyło się w październiku 1962 r. w Sao Paulo i Rio de Janeiro pod przewodnictwem A. Hollaendera z udziałem najwybitniejszych specjalistów omawianych dziedzin. Treść podzielono na dwie zasadnicze części: 1) hodowla tkanek ssaków a cytologia oraz 2) wybrane zagadnienia radiobiologii.

Bardzo cenna jest pierwsza część, w której omówiono metody hodowli tkanek, ich podstawy teoretyczne, zastosowanie do badań cytogenetycznych i interpretację cytologiczną. H. Eagle przedstawił metabolizm komórek ssaków w hodowli, wymagania co do składu środowiska i jego wpływ na wzrost i czynność hodowanych komórek. Wysoce pouczające jest zestawienie metabolizmu komórek hodowanych z metabolizmem komórek *in vivo*. P. S. Moorhead szczegółowo opisał swoją technikę hodowli leukocytów z krwi ludzkiej oraz omówił zmiany chromozomalne wywołane zakażeniem wirusowym hodowli fibroblastów i nabłonka.

Poglądowa praca A. Lima-de-Faria o replikacji kwasów dezoksyrybonukleinowych w chromozomach ludzkich jest zbyt lakoniczna i przedstawia w wielkim skrócie wyniki badań autoradiograficznych przy użyciu [^{3}H]tymidyny.

Wysoce interesujący jest natomiast referat S. M. Gartlera poświęcony zagadnieniu utrzymywania się lub zmienności niektórych cech metabolicznych i antygenowych w hodowli tkanek. Komórki osobników z brakiem dehydrogenazy 6-fosforanu glukozy, galaktozemią i akatalazemią wykazały te cechy w hodowli *in vitro*, przy czym zależnie od genotypu cechy te wykazywały charakterystyczne zmiany w czasie. Właściwości antygenowe układu A : B : 0 zanikają z czasem, antygeny transplantacyjne utrzymują się stale. Następnie Gartler podaje teoretyczne podstawy zastosowania hodowli tkanek do badania anomalii genetycznych, rozważa również mechanizmy powstawania mutacji w hodowli, zagadnienia wymiany materiału genetycznego i rekombinacji. Równie cenny i otwierający szerokie perspektywy jest artykuł L. B. Russel o doświadczalnych badaniach aberacji chromozomalnych u ssaków. Żałować może należy, że Autorka nie uwzględniła bardzo ciekawych badań nad hybrydami międzygatunkowymi (muł), a ograniczyła się wyłącznie do omówienia aberacji u myszy, które to zagadnienie zostało przedstawione wyczerpująco. Uzupełnieniem tej pracy jest obszerny referat W. J. Welshonsa o anomaliach cytologicznych u myszy. Można uznać, że wykorzystanie tego gatunku do cytogenetycznych badań doświadczalnych zostało wszechstronnie przedstawione w omawianej książce. M. A. Bender podał interesujący przegląd aberacji chromozomalnych *in vitro*, zestawiając zaburzenia występujące spontanicznie z indukowanymi promieniowaniem jonizującym. Zależności pomiędzy dawką promieniowania a przeżywaniem komórek ludzkich *in vitro* omówione zostały przez G. W. Barendsena.

Podstawy współczesnej cytodiagnostyki chromozomów człowieka przedstawione zostały w czterech referatach jej twórców. D. A. Hungerford opisał morfologię i zachowanie się chromozomów prawidłowych *in vitro*, B. M. Ślizyński — chromozomów w okresie pachytenu, J. Lejeune — ano-

malie autosomów, a E. H. Y. Chu — chromozomów płciowych. Nazwiska autorów zwalniają recenzenta od obowiązku komentowania treści tych prac, uwzględniając oczywiście fakt, że pisane były one w 1962 r. Referat S. Ohno o chromatynie płciowej stanowi cenne uzupełnienie tych artykułów.

Referaty przedstawione przez gospodarzy z Brazylii znalazły się w książce zapewne głównie ze względów kurtuazyjnych. Dotyczą one kazuistyki, obrazu chromozomów u niektórych gatunków zwierząt brazylijskich i przeglądu prac C. Bottury nad anomaliami chromozomów w schorzeniach układu krwiotwórczego. Są to przyczynki nie wnoszące istotnych danych. Dyskusja okrągłego stołu, mająca stanowić podsumowanie tej części, również niestety nie wnosi danych mogących zainteresować czytelnika spoza Ameryki Łacińskiej.

Druga część książki — wybrane zagadnienia radiobiologii — z konieczności jest bardzo wycinkowa. R. B. Settlow omówił zmiany molekularne związane z inaktywacją kwasów dezoksyrybonukleinowych przez promieniowanie ultrafioletowe, H. S. Kaplan uzupełnił te dane przeglądem swoich prac. C. Chagas oraz J. G. Nichols przedstawili w dwóch referatach wpływ promieniowań jonizujących na czynność bioelektryczną oraz pobudliwość mięśni i tkanki nerwowej. A. B. Hargraeves omówił działanie promieni jonizujących na układy enzymatyczne i enzymy, a szczególnie cholinesterazy. V. P. Bond, E. P. Cronkite i wsp. przedstawili pracę nad indukowaniem raka sutka za pomocą promieni jonizujących, a R. M. Valencia i J. L. Valencia ich wpływ na gamety i zapłodnione jaja muszki owocówki. Umieszczenie pracy C. A. Eliasa i M. Mirandy o wpływie streptomycyny na rozwój faga w paciorkowcach białych przypisać należy wspomnianej już kurtuazji wobec gospodarzy.

Reasumując, druga część pozbawiona jest myśli przewodniej i dlatego stanowi słabszą stronę książki. Najcenniejsze są wypowiedzi dyskusyjne zamieszczone tu po każdej pracy, czego brak odczuwa się w pierwszej części. Wydaje się jednak, że każdy, kto pracuje aktywnie w dziedzinie cytogenetyki człowieka i cytogenetyki doświadczalnej lub interesuje się tymi dziedzinami, winien zapoznać się z tą książką, a szczególnie jej pierwszą częścią.

Przemysław Czerski

A. Sollberger: BIOLOGICAL RYTHM RESEARCH. Elsevier Publ. Co., Amsterdam-London-New York 1965; str. 462, cena Dfl. 70.-, sh. 140, DM 78.-

Książka stanowi szeroki przegląd osiągnięć w dziedzinie badania rytmów biologicznych. Jest ona jednym z nielicznych dotychczas syntetycznych opracowań, przedstawiających to zagadnienie z punktu widzenia różnych dyscyplin nauk biologicznych.

Treścią książki są zarówno opis i klasyfikacja rytmów biologicznych, sugestie dotyczące metod ich badania i rejestracji, jak i próby wyjaśnienia mechanizmu oraz perspektywy wykorzystania znajomości tych zjawisk w praktyce, zwłaszcza medycznej.

We wstępnym rozdziale przedstawił Autor podstawowe pojęcia dotyczące periodyczności funkcji biologicznych. Część I książki dotyczy problemu spontanicznych, endogennych oscylacji funkcji. Autor w przystępny sposób, popierając rozważania teoretyczne bardzo licznymi przykładami, wprowadza czytelnika w zagadnienie regulacji rytmów endogennych, ze szczególnym uwzględnieniem udziału w niej mechanizmów sprzężenia zwrotnego. Rozdział poprzedzony jest ogólnym omówieniem podstawowych pojęć cybernetycznych, wykorzystywanych w dalszej części książki. Szerzej omawia Autor zagadnienie „zegara biologicznego" — poczucia trwania czasu i orientacji w czasie, specjalny nacisk kładąc na opis i krytykę metod stosowanych do badań tego zagadnienia. Tę część książki zamyka wprowadzenie do „chronopatologii" (zaburzeń periodyczności zjawisk biologicznych) oraz omówienie onto- i filogenezy rytmów biologicznych.

Część II poświęcona jest rytmicznym zmianom w środowisku zewnętrznym, które mogą wywierać wpływ na rytmy endogenne oraz próbom wyjaśnienia mechanizmu ich działania.

Szczególnej uwagi godna jest część III książki, poświęcona matematycznej i statystycznej analizie zjawisk rytmicznych. Przedmiot potraktowany został przez Autora nader szeroko, w sposób który, nie zmniejszając zwartości treści książki, czyni ją zrozumiałą także dla niespecjalisty w tej dziedzi-

nie. Zyskuje on tu znajomość ogólnych zasad matematycznego i statystycznego traktowania zjawisk periodycznych, które czytelnik-biolog może wykorzystać do opracowywania wyników badań rytmów biologicznych.

Ostatnia, IV część książki poświęcona jest w zasadzie rytmom egzogennym. Znaczną część treści stanowi omówienie rytmów dobowych, miesięcznych (księżycowych), sezonowych itd. oraz znaczenia ich znajomości dla wyjaśnienia wielu zjawisk stanowiących przedmiot badań zoologii, botaniki, medycyny itp.

Książka opatrzona jest licznymi wykresami oraz bardzo obszernym (124 strony) zestawieniem piśmiennictwa przedmiotu. Napisana jest w przejrzysty sposób, klasyfikuje bogaty materiał badań dotyczących rytmów biologicznych oraz szeroko uwzględnia matematyczne aspekty analizy tych zjawisk.

Stanisław Kozłowski

R. W. Bailey: OLIGOSACCHARIDES. Pergamon Press, Oxford 1965; str. 179, cena 60 s.

Jako czwarty tom Międzynarodowej Serii Monografii, zajmujących się czystą i stosowaną biologią, R. W. Bailey (Nowa Zelandia), jeden z najlepszych znawców przedmiotu, ogłosił w formie monograficznej zestawienie dotąd poznanych oligosacharydów.

We wstępnym rozdziale znajdujemy proponowane przez Autora dane, dotyczące zasięgu, klasyfikacji i nomenklatury tych związków, które zaliczyć można do klasy oligosacharydów, a których objęcie bynajmniej nie jest łatwe i często kontrowersyjne wobec dużej i wciąż rosnącej liczby tych związków o różnorodnym planie budowy. Jako główne zasady klasyfikacji proponuje Bailey następujący podział: a) zbudowane z prostych jednocukrowców; b) heterogenne w odniesieniu do jednostek jednocukrowców; c) zawierające aminocukry i d) zawierające kwasy uronowe. Porządkując nomenklaturę oligosacharydów Autor stosuje w książce obok nazw potocznych (trywialnych) jeszcze i nazwy skrócone (wg. Whelana), a także pełne definitywne nazwy, oparte o brytyjsko-amerykańskie prawidła (p. Editorial Report, *J. Chem. Soc.* 5108, 1952).

Drugi rozdział poświęcony jest występowaniu w przyrodzie oligosacharydów jako produktów naturalnych oraz występowaniu oligosacharydów jako produktów rozlicznych syntez, stosujących zarówno metody chemiczne jak enzymatyczne. W następnym rozdziale omawia Autor metody preparatyki i właściwości oligosacharydów oraz w przypadkach, gdzie dostępne są wystarczające dane, dowody proponowanej struktury. Rozdział ten zawiera ponadto ocenę metod, stosowanych dla ustalenia struktury oligosacharydów. Następne rozdziały zawierają uporządkowane zestawienia dotąd poznanych oligosacharydów z ich nazwami oraz dane, odnoszące się do skręcalności optycznej, punktu topnienia, jak i właściwości niektórych pochodnych, jak octanu, fenyloosazonu i in.; wreszcie w zestawieniu tym przy naturalnych oligosacharydach podane są źródła i występowanie w przyrodzie. Książkę zamyka klasyfikowany indeks dotąd poznanych oligosacharydów, uporządkowany alfabetycznie.

Książka przedstawia dużą wartość dla chemików, chcących zapoznać się z najnowszymi osiągnięciami chemii cukrowców, dla biologów, biochemików oraz lekarzy; publikację tę można ocenić jako udaną próbę uporządkowania naszych wiadomości o związkach zbudowanych z kilku cegiełek cukrowcowych.

Edmund Mikulaszek

W. E. Ribelin and J. R. McCoy: THE PATHOLOGY OF LABORATORY ANIMALS. Charles C. Thomas, Publ., Springfield (Ill.) 1965; str. 436; cena $ 14.75.

Dzieło to jest zbiorem referatów i głosów w dyskusji wygłoszonych na konferencji poświęconej patologii zwierząt doświadczalnych, zorganizowanej przez Nowojorską Akademię Medycyny.

Książka jest ułożona według dwojakiego klucza. Jedna jej część jest poświęcona zmianom chorobowym w niektórych układach. Należą tu rozdziały o chorobach układu ruchu, układu siateczk-

kowo-śródbłonkowego, oddechowego, dokrewnego, nerek, serca i wątroby. W drugiej części są omówione naturalne choroby chomika, szczura, myszy, królika i małpy, a także samoistne i wywołane nowotwory świnki morskiej. Wymienionym tu zwierzętom poświęca się najwięcej uwagi. Pies i kot są przedmiotem nielicznych wzmianek, redaktorzy uważają bowiem, że dostatecznie dużo wiadomości na temat tych zwierząt można znaleźć w podręcznikach weterynarii.

Na charakterze książki niewątpliwie odbija się fakt, że jest to zbiór referatów, siłą rzeczy ograniczonych w czasie. W druku odnosi się wrażenie lakoniczności. Wszystkie wiadomości są podane bardzo zwięźle, telegraficznie. Ponieważ jednak książka liczy 436 stronic, wiadomości tych jest bardzo wiele. Każdy rozdział jest uzupełniony dobrze dobranym i nowoczesnym piśmiennictwem.

Szata graficzna książki jest na bardzo wysokim poziomie.

Każdy badacz, mający do czynienia ze zwierzęciem doświadczalnym, z przyjemnością i pożytkiem weźmie tę książkę do ręki. O znaczeniu znajomości naturalnych chorób zwierząt doświadczalnych nie trzeba przekonywać, a monografii na ten temat jest niewiele.

Stefan Kruś

J. G. Shaffer, W. H. Shlaes and R. A. Radke: AMEBIASIS, A8BIOMEDICAL PROBLEM. Charles C. Thomas Publ., Springfield (Ill.) 1965; str. 172, cena $ .50.

Jest to bardzo wyczerpująca monografia poświęcona amebiazie. Autorzy omawiają w niej historię badań nad amebiazą, etiologię (cechy i cykl pełzaków), patogenezę i anatomię patologiczną amebiazy, epidemiologię, diagnostykę laboratoryjną, symptomatologię kliniczną i leczenie.

W sumie jest to książka niezmiernie pożyteczna, zarówno dla klinicystów, jak i patologów i epidemiologów. Ze względu na rosnący kontakt polskich lekarzy z chorobami tropikalnymi, monografię tę można gorąco polecić czytelnikowi polskiemu.

Autorzy bardzo szeroko opisują zmiany morfologiczne, obserwowane w amebiazie. Niestety niektóre mikrofotografie są mało czytelne i to jest jedyna chyba usterka tego wydawnictwa.

Stefan Kruś

G. I. C. Ingram and J. Richardson, ANTICOAGULANT PROPHYLAXIS AND TREATMENT. Charles C. Thomas Publ., Springfield (Ill.), 1965; str. XXI+247; cena $ 8.75.

Autorzy książki na podstawie najnowszych zdobyczy badań doświadczalnych na zwierzętach oraz wyników obserwacji klinicznych u ludzi omawiają zagadnienia profilaktyki i leczenia zakrzepów.

Pierwsze rozdziały książki wprowadzają w zagadnienie od strony teoretycznej. Autorzy dokonują podziału znanych leków przeciwzakrzepowych na dwie grupy: grupę heparyny, która wykazuje bezpośrednie działanie także *in vitro*, i grupę licznych pochodnych układu kumaryny oraz indandionu, które działają na układ krzepnięcia tylko *in vivo*. W oparciu o schemat krzepnięcia krwi zostały przedstawione własności przeciwzakrzepowe poszczególnych leków i częściowo znane mechanizmy ich działania. W dalszych rozdziałach autorzy omawiają najnowsze poglądy na przyczyny i mechanizmy tworzenia się zakrzepów i zatorów. Obok bardziej uchwytnych i dających się dłużej obserwować zmian anatomicznych w ścianach naczyń lub krążeniu, obok zmian w składnikach układu krzepnięcia lub fibrynolizy zwrócona zostaje uwaga na krótkotrwałe wymykające się spod obserwacji działanie bodźców emocjonalnych, które poprzez doświadczalnie stwierdzony wpływ adrenaliny odbijają się na układzie krzepnięcia i mogą doprowadzić do wystąpienia zakrzepu. Omówiony zostaje przy tym wpływ leków przeciwzakrzepowych, oddzielnie heparyny i grupy pochodnych kumaryny i indandionu, na tworzenie się wewnątrznaczyniowych zakrzepów.

Ze względu na to, że wyniki prac doświadczalnych *in vitro* lub *in vivo* na zwierzętach tylko z wielką ostrożnością mogą być wykorzystywane do wnioskowania o reaktywności organizmu ludzkiego, druga część książki dotyczy wyłącznie obserwacji klinicznych. W części tej omówiono szereg zespo-

łów chorobowych, którym towarzyszy niebezpieczeństwo tworzenia zakrzepów. Przytoczono wyniki obserwacji grup chorych nieleczonych i leczonych preparatami przeciwzakrzepowymi, zestawiając liczbę zejść śmiertelnych, liczbę remisji całkowitych lub częściowych po leczeniu. Zebrany materiał dowodowy licznych autorów wskazuje na olbrzymie praktyczne znaczenie umiejętnie stosowanych leków przeciwzakrzepowych w terapii różnych zespołów chorobowych. W podsumowaniu każdego rozdziału autorzy zestawiają wskazania i przeciwskazania do stosowania antykoagulantów. Końcowe rozdziały II części książki poświęcone są niektórym rzadziej spotykanym stanom chorobowym. Szczegółowo została omówiona sprawa odwłóknienia krwi — geneza, diagnostyka i terapia. Znaczenie antykoagulantów zostało wyjaśnione przy występowaniu nocnej napadowej hemoglobinurii oraz przy fenomenie Schwarzmana.

Część III książki omawia szczegółowo terapię przeciwzakrzepową: wielkość dawek, jednorazowe lub wielokrotne zastosowanie leków, ocenia szybkość efektów i ich natężenie, sprawę kumulowania się leków i reakcje organizmu na odstawienie. Szczególnie dokładnie zostały przedstawione sprawy niebezpieczeństw związanych z przedawkowaniem leków i pojawieniem się ubocznych toksycznych reakcji.

Ostatni rozdział daje przegląd i krytyczną ocenę metod i ich różnych modyfikacji, stosowanych w laboratoriach dla diagnostyki powikłań zakrzepowych lub przedawkowania antykoagulantów.

Książka jest cenną pozycją, wiąże bowiem zagadnienia teoretyczne i wyniki prac eksperymentalnych z praktycznymi potrzebami nowoczesnej medycyny w zakresie lecznictwa i profilaktyki chorób zakrzepowych. Liczne cytaty z piśmiennictwa dają bogaty przegląd prac doświadczalnych i klinicznych w zakresie krzepnięcia krwi do r. 1965. Książka powinna zainteresować klinicystów stykających się z problemami zaburzeń krzepnięcia i ich leczeniem, farmakologów zajmujących się działaniem leków przeciwzakrzepowych oraz fizjologów i biochemików pracujących nad czynnikami układu krzepnięcia krwi.

Maria Gumińska