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ACIDIC TRYPSIN INHIBITOR FROM BOVINE PANCREAS. STABILITY OF THE INHIBITOR-TRYPSIN COMPLEX

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1. Isolation of the trypsin inhibitor from trichloroacetic acid extract of pancreas is described. 2. The inhibitor preparation inactivates trypsin at a weight ratio of 1:5 and possesses the properties of the Kazal inhibitor. 3. The trypsin-inhibitor complex is stable at pH 7 provided that no free trypsin is present in the medium. 4. Chymotrypsin has no effect on the free inhibitor but destroys the inhibitor when it is bound with trypsin.

Two polypeptide trypsin inhibitors soluble in trichloroacetic acid have been isolated from bovine pancreas. A basic inhibitor was isolated by Kunitz & Northrop (1936), and an acidic one by Kazal, Spicer & Brahinsky (1948). The latter, in contrast to the former, is digested by pepsin, is inactive toward chymotrypsin and plasmin, and inhibits trypsin without previous preincubation.

Wilimowska-Pelc & Mejbaum-Katzenellenbogen (1969) from the trichloroacetic acid extract of bovine pancreas obtained by ammonium sulphate fractionation a preparation, 10 μg of which inhibited 1 μg of trypsin and which exhibited the properties of the Kazal inhibitor. In the present work, further purification of the inhibitor was achieved and the purified preparation was used in the experiments on the stability of the trypsin-trypsin inhibitor complex, and the effect of chymotrypsin.

MATERIALS AND METHODS

Preparation of the inhibitor. The first steps of the procedure were carried out as described by Wilimowska-Pelc & Mejbaum-Katzenellenbogen (1969) except that heating of the trichloroacetic acid extract at 80°C was omitted. Frozen bovine pancreas was homogenized with 5% trichloroacetic acid, the extracted protein was concentrated by the tannin-caffeine procedure of Mejbaum-Katzenellenbogen (1959), precipitated by 0.6 ammonium sulphate saturation at pH 5, then, instead of the second ammonium sulphate precipitation which resulted in considerable

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losses of inhibitor, CM-cellulose column chromatography was employed. For this purpose, the ammonium sulphate-precipitated protein was dissolved in 0.01 M-acetate buffer, pH 5, and dialysed against 0.01 M-acetate buffer, pH 5, at -5°C. The sediment which appeared during dialysis was removed by centrifugation and the supernatant (1 - 1.5% of protein) was applied to the CM-cellulose column equilibrated with 0.01 M-acetate buffer. The elution was performed with the same buffer at a concentration gradient of 0.01 - 0.2 M (Fig. 1). The fractions containing the inhibitor were
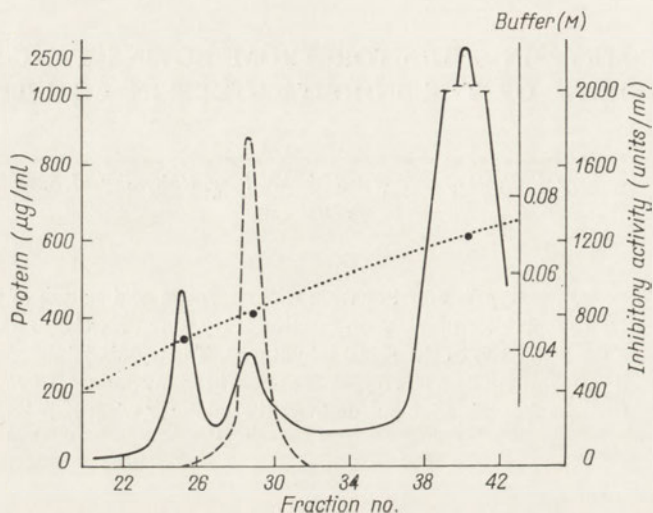

Fig. 1. CM-cellulose column chromatography of proteins precipitated at 0.6 ammonium sulphate sat. from the trichloroacetic acid extract of pancreas. The protein, 1.05 g, was applied to the column (40 × 3 cm) and eluted with a 0.01 - 0.2 M gradient of acetate buffer, pH 5, fractions of 20 ml being collected at a rate of 60 ml/h. Trypsin inhibitory activity was determined by the peptide test. ————, protein; — — — trypsin inhibitory activity; ···, concentration gradient.

pooled, acetone was added to 80% concentration at -5°C and the precipitate formed was washed twice with acetone at the same temperature. The preparation was dissolved in water, desalted on Sephadex G-25 column and precipitated again with acetone. The purification of the inhibitor at the successive steps of its isolation is presented in Table 1. From 1 kg of pancreas, 3 mg of the inhibitor was obtained, this yield being similar to that reported by Cerwinsky, Burck & Grinnan (1967).

Trypsin inhibitory activity. This was determined by the peptide test based on the tannin method (Mejbaum-Katzenellenbogen, Wilusz & Polanowski, 1966), and the method of Kunitz (1947) using in both cases casein as substrate. One unit of the inhibitor is defined as the capacity to inactivate 1 µg of trypsin; in the Kunitz test the amount of inactivated trypsin was calculated from the standard curve (Fig. 2).

The effect of chymotrypsin on the inhibitor was studied in 0.01 M-CaCl$_2$ - 0.05 M-borate buffer, pH 7, under toluene. The samples, 2 ml, contained 20 µg of the inhibitor preparation and 8 or 16 µg of chymotrypsin. After 24 h at 30°C, an equal

Table 1

*Isolation of the trypsin inhibitor from bovine pancreas*

The inhibitory activity was assayed by the peptide test. One unit of the inhibitor is defined as the capacity to inactivate 1 μg of trypsin.

Purification step	Protein (mg/kg of pancreas)	Activity (units/kg of pancreas)	Spec. act. (units/mg protein)	Yield (%)	Purification factor
1. Trichloroacetic acid extract	1636	84 200	50	100	1
2. Concentration by the tannin-caffeine procedure	1014	55 770	60	66	1.1
3. Ppt. at 0.6 $(NH_4)_2SO_4$ sat.	354	35 400	100	48	2
4. CM-cellulose chromatography (peak *II*, see Fig. 1)	3.5	17 500	5000	20	100

volume of 5% trichloroacetic acid was added. The mixture was heated for 5 min at 80°C, centrifuged and the trypsin inhibitory activity was determined in the supernatant.

The stability of the trypsin-trypsin inhibitor complex was studied by the method of Laskowski & Wu (1953); trypsin was incubated with different amounts of the inhibitor in 0.01 M-$CaCl_2$ - 0.05 M-borate buffer, pH 7, under toluene. Samples

Fig. 2. Standard curve for crystalline trypsin activity measured by the Kunitz test. The incubation mixture, 2 ml, contained 10 mg of casein and increasing amounts of trypsin. After 20 min at 35.5°C, 3 ml of 5% trichloroacetic acid was added, and the extinction at 280 nm was read.
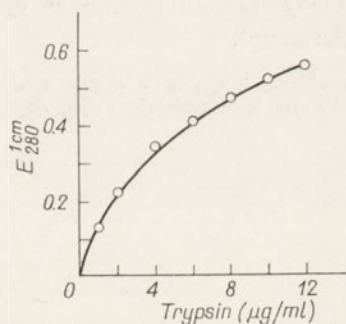
of the incubation mixture were periodically withdrawn and trypsin activity was determined and compared with the activity of trypsin which had been incubated under the same conditions but without the inhibitor. To check for the possible inactivation of the inhibitor, to the simultaneously withdrawn samples of the incubation mixture an equal volume of 5% trichloroacetic acid was added to dissociate the complex with simultaneous precipitation of trypsin. In the supernatant, the inhibitory activity was determined.

Protein was determined by the tannin micromethod (Mejbaum-Katzenellenbogen, 1955).

Paper electrophoresis was carried out in veronal-acetate buffer, pH 9, and in acetate buffer, pH 4.7, at 220 V for 5 h. About 100 µg of protein was applied on strips (28×3 cm) of Whatman no. 1 paper. The protein was stained with bromophenol blue in the tannin reagent (Mejbaum-Katzenellenbogen & Dobryszycka, 1959).

Reagents. Tannic acid was a U.S.S.R. product, checked for the presence of pentadigalloylglucose by extraction with ethyl acetate according to Armitage *et al.* (1961). Soluble casein, light white, was from British Drug Houses (Poole, Dorset, England). Trypsin was prepared from bovine pancreas by the method of Northrop & Kunitz (cf. Laskowski, 1955); the preparation was crystallized twice and showed no chymotrypsin activity toward the ethyl ester of tyrosine (Serva, Heidelberg, West Germany). Chymotrypsin was prepared from chymotrypsinogen A by the method of Kunitz & Northrop (1936). Soya-bean inhibitor was obtained as described by Polanowski & Olichwier (1969); it inhibited trypsin when applied at a ratio of 1:1. Whatman carboxymethyl-cellulose, CM-70, was a product of Balston Ltd. (Maidstone, Kent, England). Other chemicals were reagent grade products of Polskie Odczynniki Chemiczne (Gliwice, Poland).

RESULTS

Inhibition of trypsin by the obtained preparation, measured both by the Kunitz test (Fig. 3) and the peptide test, was proportional to the amount of inhibitor added; 0.2 µg of the preparation inhibited the activity of 1 µg of trypsin. As the molecular weight of trypsin is 24 000, and assuming that the inhibitor reacts with trypsin at an equimolar ratio, it may be supposed that its molecular weight is about 5000. This assumption is supported by the fact that the purified preparation diffused slowly through cellophane membranes (Kalle A. G., Wiesbaden-Biebrich, West Germany).

On paper electrophoresis at pH 9 and 4.7 the inhibitor preparation migrated toward the cathode (Fig. 4).

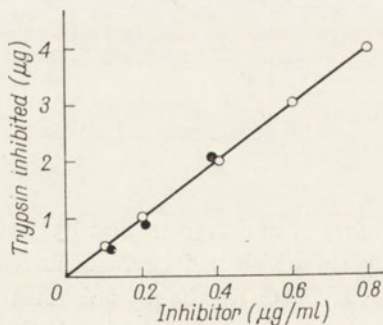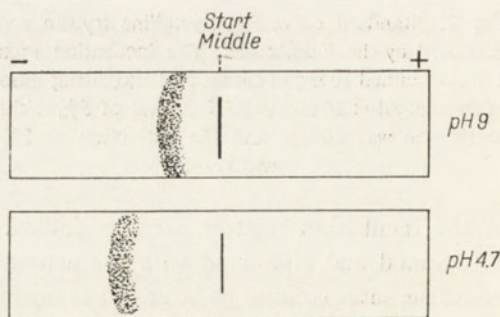Fig. 3   Fig. 4

Fig. 3. Effect of inhibitor concentration on the amount of inhibited trypsin. The Kunitz test was applied; the incubation mixture contained in 1 ml 5 mg of casein, the indicated amount of the inhibitor preparation and: ●, 4 µg of trypsin; or ○, 2 µg of trypsin.

Fig. 4. Paper electrophoresis of the purified inhibitor preparation in veronal-acetate buffer, pH 9, and in acetate buffer, pH 4.7.

The purified inhibitor preparation, similarly as the preparation obtained previously (Wilimowska-Pelc & Mejbaum-Katzenellenbogen, 1969) inhibited selectively trypsin. Although the trichloroacetic acid extract of pancreas inhibited also chymotrypsin, the ·protein concentrated by the tannin-caffeine procedure proved to be inactive. Thus it may be supposed that only one of the two polypeptide inhibitors became released by caffeine from the complex with tannin.

The inhibitor exhibited considerable thermostability. Heating of the trichloroacetic acid extract for 15 min at 80°C did not inactivate the inhibitor. No loss of activity was observed after boiling of the purified preparation for 10 min in aqueous solution; the activity was also unaffected by alkali, even after storage for three days in 0.01 N-NaOH at room temperature.

Complex of the inhibitor with crystalline trypsin preparation free of chymotrypsin was stable provided that the amount of the inhibitor present was sufficient to inhibit completely the activity of trypsin. The stability of the complex at different weight ratios of the two components in the incubation mixture is presented in Fig. 5.
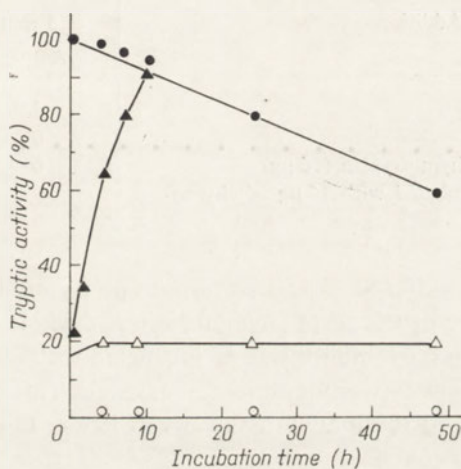

Fig. 5. The stability of the trypsin-trypsin inhibitor complex. A solution containing trypsin and inhibitor in 3 ml of 0.01 M-CaCl$_2$ - 0.05 M-borate buffer, pH 7, was incubated at 37°C. At the indicated time intervals, samples of 0.1 ml were withdrawn and tryptic activity was determined according to Kunitz (1947). Conditions: 200 µg of trypsin, and: ○, 65 µg of pancreatic inhibitor (weight ratio 0.32:1, excess of inhibitor); ▲, 32 µg of pancreatic inhibitor (weight ratio 0.16:1, excess of trypsin); △, 160 µg of soya-bean inhibitor (weight ratio 0.8:1, excess of trypsin); ●, trypsin control.

In the presence of an excess of the inhibitor (0.31 µg/1 µg trypsin), the tryptic activity did not appear even after 48 h of incubation, whereas when free trypsin was present (0.16 µg of inhibitor/1 µg of trypsin) the tryptic activity gradually increased and after 8 hours practically no inhibition was observed. For comparison, the stability of the complex of soya-bean inhibitor with trypsin was also studied; in the presence of 0.8 µg of inhibitor/1 µg trypsin the complex was stable although free trypsin was present in the incubation mixture.

When the inhibitor was incubated with crystalline trypsin, the whole inhibitory activity could be always recovered after removal of trypsin with trichloroacetic acid. When, however, a commercial trypsin preparation (Merck, Darmstadt, West Germany, batch no. 62211) was applied which, as demonstrated by the test with tyrosine ethyl ester, contained some chymotrypsin activity, the trypsin inhibitory activity could not be demonstrated in the supernatant after addition of trichloroacetic acid. Therefore the effect of chymotrypsin both on the complex and the inhibitor alone was studied (Table 2). After 20 hours of incubation of crystalline trypsin

Table 2

Effect of chymotrypsin on the stability of the trypsin-trypsin inhibitor complex

The incubation mixture contained in 2 ml of 0.01 M-$CaCl_2$ - 0.05 M-borate buffer, pH 7, 80 µg of trypsin and the indicated additions. After 5 min or 20 h of incubation, samples of 0.1 ml were withdrawn and trypsin activity was determined according to Kunitz (1947).

Addition	Incubation time	
	5 min	20 h
	Active trypsin (µg/0.1 ml)	
None	4.0	3.4
Inhibitor (20 µg)	0	0
Inhibitor (20 µg) and chymotrypsin (16 µg)	0	3.8
Inhibitor (20 µg) preincubated with 16 µg of chymo- trypsin	0	3.8

with an excess of the inhibitor (0.25 µg/1 µg trypsin) no tryptic activity was observed, whereas in the presence of 0.2 µg of chymotrypsin the whole tryptic activity reappeared after 20 h. When the inhibitor was preincubated with chymotrypsin for 24 h, the trypsin inhibitory activity was found to be unaltered. This indicates that chymotrypsin was active toward the inhibitor only when it was bound with trypsin but did not digest the free inhibitor.

DISCUSSION

Laskowski & Wu (1953) observed that the complex of Kazal inhibitor with trypsin became slowly activated on standing. They called this phenomenon "temporary inhibition" and demonstrated that, while trypsin was liberated from the complex, the inhibitor was slowly destroyed.

In our experiments, when trypsin was not completely bound by the inhibitor and some free trypsin was present in the incubation mixture, within 8 hours all the inhibitor became destroyed and all trypsin activity reappeared. On the other hand, when the incubation mixture contained an excess of the inhibitor, the complex was stable up to 48 h of incubation. Possibly, the digestion of the inhibitor might occur after a still longer time of incubation. Burck, Hamill, Cerwinsky & Grinnan (1967) demonstrated that in the presence of an excess of the Kazal inhibitor the tryptic activity appeared as late as after 5 days of incubation.

Another characteristic feature of the Kazal inhibitor is that it is present also in the pancreatic juice. Fritz, Hüller, Wiedemann & Werle (1967) demonstrated that, in the pancreatic juice and pancreas of the dog, there is a correlation between the content of the inhibitor and that of trypsinogen, and they suggested that the inhibitor might regulate the activation of pancreatic proenzymes.

In the present work it has been demonstrated that, if small amounts of chymotrypsin are present in the trypsin preparation, it is not possible to obtain a stable complex even in the presence of an excess of inhibitor; thus chymotrypsin appears to facilitate the liberation of the inhibitor from the complex with trypsin. The inhibitor itself does not undergo digestion by chymotrypsin although it contains chymotrypsin-labile bonds (Greene & Giordano, 1969). Probably the secondary structure of the inhibitor makes these bonds inaccessible to the action of chymotrypsin. Rigbi & Greene (1968) have observed that during the reaction of the Kazal inhibitor with trypsin, the bond formed by arginine at position 18 and isoleucine at position 19 undergoes cleavage. As a result, some changes in the structure of the inhibitor may occur which would expose the chymotrypsin-susceptible bonds. A similar mechanism may occur in the pancreatic juice, in which small amounts of chymotrypsin could accelerate the liberation of trypsin from the complex with the inhibitor. This could form a specific mechanism providing trypsin for activation of pancreatic proenzymes.

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KWAŚNY INHIBITOR TRYPSYNY Z TRZUSTKI BYDLĘCEJ.
TRWAŁOŚĆ KOMPLEKSU INHIBITORA Z TRYPSYNĄ

Streszczenie

1. Opisano metodę otrzymywania inhibitora trypsyny z trójchlorooctowych ekstraktów trzustek bydlęcych.

2. Inhibitor hamował trypsynę w stosunku 1:5 i posiadał właściwości charakterystyczne dla inhibitora Kazala.

3. Kompleksy inhibitora z trypsyną w pH 7 są trwałe w wypadku nieobecności wolnej trypsyny w środowisku.

4. Chymotrypsyna nie trawi inhibitora, ale przyśpiesza proces jego trawienia w kompleksie z trypsyną.

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NUCLEOSIDE DIPHOSPHATE SUGAR PYROPHOSPHORYLASES OF *SHIGELLA FLEXNERI* AND *ESCHERICHIA COLI*

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The presence of nucleoside diphosphate sugar pyrophosphorylases (the EC 2.7.7. group of nucleotidyltransferases) synthetizing ADPglucose, CDPglucose, GDPglucose, dTDPglucose and UDPglucose was demonstrated in cell-free extracts from *Shigella flexneri* 2a. Partial separation of these enzymes was performed by gel filtration on Sephadex G-200. The elution volumes of individual enzymes in extracts of *E. coli* and *Sh. flexneri* were similar.

The occurrence of a variety of pyrophosphorylases differing in the specificity with respect both to nucleoside and sugar residues has been known since the studies of Bernstein & Robbins (1965). Chojnacki, Sawicka & Korzybski (1968b) extended these studies to the *Salmonella* genus, and they obtained more extensive separation of these enzymes by gel filtration on Sephadex G-200. The kind of the pyrophosphorylases might well be a reflection of the actual composition of the O-antigenic polysaccharide in a given species, as in the biosynthesis of certain specific sugars only specified nucleotide coenzymes are involved. Thus 3,6-dideoxyhexose residue arises *via* the cytidine coenzymes and in the biosynthesis of rhamnose, thymidine nucleotide is involved (Robbins, Bray, Dankert & Wright, 1967).

This paper presents the results of the studies concerning the first step of the biosynthesis of O-antigenic polysaccharide, i.e. the reactions catalysed by the pyrophosphorylases responsible for the formation of ADPglucose, CDPglucose, dTDPglucose and UDPglucose (the EC 2.7.7. group). Bernstein & Robbins (1965) and Chojnacki *et al.* (1968b) reported that separate enzymes were responsible for the formation of each nucleoside type of nucleoside diphosphate sugar, and that the molecular weights of a given enzyme were similar in *E. coli* and *S. typhimurium*. The studies on the pyrophosphorylases in *Shigella flexneri* were undertaken to re-examine the previous data indicating that TDPglucose was the only nucleoside diphosphate sugar formed in this species (Janczura, Załęska & Chojnacki, 1971).

MATERIALS AND METHODS

Bacterial cultures of *E. coli* Hfr and *Shigella flexneri* 2a were obtained from the Department of Bacteriology (State Institute of Hygiene, Warszawa). They were cultivated as described previously (Janczura *et al.*, 1971).

Chemicals. Blue dextran 2000, Sephadex G-25 and Sephadex G-200 were from Pharmacia (Uppsala, Sweden). Cytochrome *c* was from Polfa (Warszawa, Poland). The origin of other chemicals, or the method of their preparation was described by Chojnacki *et al.* (1968b).

Analytical. The assay of phosphorus, protein and paper chromatography were performed as described by Chojnacki *et al.* (1968b).

Enzyme preparations. The bacterial cells harvested in middle-logarithmic phase were washed with 0.145 м-NaCl, suspended in 0.145 м-NaCl - 0.005 м-tris-HCl buffer, pH 7.6, and sonicated at about 10 kc for 4 min with intervals in a cooled vessel. The sonicated suspensions were centrifuged at 20 000 *g* for 20 min and the clear supernatant was collected for determination of enzyme activity.

The estimation of the activity of pyrophosphorylases was performed essentially as described by Chojnacki *et al.* (1968b). The assay of ^{32}P-labelled nucleoside diphosphate sugars adsorbed on charcoal was performed in a Packard scintillation spectrometer using Cerenkov's radiation as described by Chojnacki & Matysiak (1971).

Gel filtration. The columns (1 × 20 cm) of Sephadex G-25 were used for removing low-molecular-weight substances from bacterial extracts. The fractionation of enzymes of bacterial extracts was performed on a 2 × 80 cm column of Sephadex G-200 or on a 0.8 × 14 cm column of Sepharose 4B. Blue dextran and cytochrome *c* were used for calibrating the columns. The gels were equilibrated with 0.145 м-NaCl - 0.005 м-tris-HCl buffer, pH 7.6. The same solution was used for fractionation of bacterial extracts.

RESULTS

The rates of formation of various nucleoside diphosphate sugars from nucleoside-5'-triphosphates and radioactive [^{32}P]glucose-1-phosphate in the extracts from *E. coli* and *Sh. flexneri*, exhibited different patterns (Table 1). Whereas in *E. coli* all studied nucleoside-5'-triphosphates gave rise to the formation of radioactive nucleoside diphosphate sugars, in *Shigella flexneri* none of the nucleoside diphosphate sugars was formed in the amount as high as in *E. coli*. This was also true for bacterial extracts from which low-molecular-weight substances had been removed by passing the extracts through Sephadex G-25. As shown in Table 1, gel filtration through Sephadex G-25 resulted in an only slight increase of the rate of formation of some nucleoside diphosphate sugars in *E. coli*.

The experiments in which the proteins of *Shigella flexneri* extracts were fractionated on Sephadex G-200 have demonstrated unequivocally the formation of

Table 1

Formation of nucleoside diphosphate sugars in extracts of Shigella flexneri *and* Escherichia coli

The reaction mixture contained the following components in a total volume of 0.6 ml: [^{32}P]glucose-1-phosphate, 0.05 μmole (2.7 × 10^4 c.p.m.); the indicated nucleoside-5′-triphosphate, 0.1 μmole; tris-HCl buffer, pH 7.6, 20 μmoles; MgCl$_2$, 6 μmoles and 0.02 ml of crude extract or 0.03 ml of the eluate from Sephadex G-25 (30 μg of protein in each case). Time of incubation: 30 min. The results are expressed in c.p.m. of ^{32}P adsorbed on charcoal.

Incubation with ...	0	ATP	CTP	GTP	UTP	dTTP
Crude extract						
Sh. flexneri	202	146	152	128	181	205
E. coli	382	2237	2968	3840	2132	1413
Eluate from Sephadex G-25						
Sh. flexneri	208	140	121	125	306	203
E. coli	397	3526	3639	3952	2116	1483

nucleoside diphosphate sugars from all nucleoside-5′-triphosphates (Fig. 1a). The molecular size of enzymic proteins was similar to that found in *E. coli* (Fig. 1b) as judged from their elution volumes.

The discrepancy between the results of the assays of various pyrophosphorylases obtained in unfractionated extracts of *Sh. flexneri* and in eluates from Sephadex G-200 might have been due to the presence in the former of a high-molecular inhibitory factor that counteracted the formation of nucleoside diphosphate sugars. In the eluate from Sephadex G-200 containing the proteins of the *Shigella flexneri* extract this factor should presumably be present between the 75th and 85th ml (cf. Fig. 1a). To check this suggestion the protein fractions from the same experiment that catalysed the formation of given nucleoside diphosphate sugar (fractions between 45th and 55th ml) were mixed with an equal volume of the fraction eluted between 75th and 85th ml. It was found that the formation of various nucleoside diphosphate sugars dropped then again to zero. Paper chromatography of the reaction mixtures containing the 75 - 85 ml fraction demonstrated a complete cleavage in 15 min of [^{32}P]glucose-1-phosphate with the formation of orthophosphate thus preventing its entering the reaction catalysed by the pyrophosphorylase. The results of this experiment may explain the discrepancy between the results of the assay of pyrophosphorylases in unfractionated and fractionated bacterial extracts. They demonstrate that the presence of a phosphatase in crude extract of *Sh. flexneri* prevents the formation of nucleoside diphosphate sugars by splitting glucose-1-phosphate.

Whereas the dTDPglucose pyrophosphorylase could easily be separated from other enzymes by gel filtration on Sephadex G-200, the other pyrophosphorylases were eluted in one group, except UDPglucose pyrophosphorylase in *Sh. flexneri*. Attempts to obtain better separation of these enzymes using Sepharose 4B were unsuccessful.
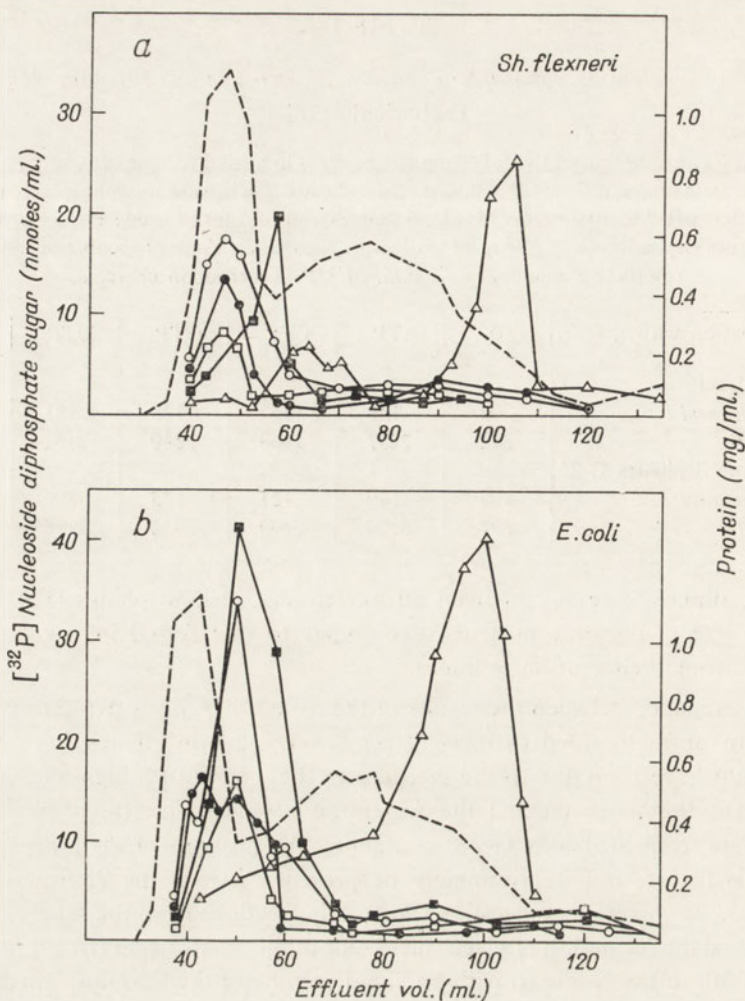
Fig. 1. Fractionation of nucleoside diphosphate glucose pyrophosphorylases of *Shigella flexneri* (*a*) and *Escherichia coli* (*b*) by gel filtration on Sephadex G-200. 20000 *g* supernatants of sonicated suspensions of bacterial cells (2 ml) were applied to a 2 × 80 cm column of Sephadex G-200 and eluted with 0.145 M-NaCl - 0.005 M-tris-HCl buffer, pH 7.5. Fractions of 3 ml were collected. Pyrophosphorylases of: ○, ADPglucose; □, CDPglucose; ●, GDPglucose; ■, UDPglucose; △, dTDPglucose. All activities are expressed in nmoles of synthetized nucleoside diphosphate sugar per 1 ml of eluate. — — —, Amount of protein (mg/ml). The elution volume of Blue dextran was 38 ml and that of cytochrome *c*, 115 ml.

DISCUSSION

Preliminary experiments on the pyrophosphorylases in extracts from *Shigella flexneri* failed to detect any enzymic activity or demonstrated only weak activity of dTDPglucose pyrophosphorylase (Chojnacki, Sawicka, Janczura, Załęska & Korzybski, 1968a; Janczura *et al.*, 1971). On the other hand, these enzymes were well

detectable in *E. coli* or *S. typhimurium* (Chojnacki *et al.*, 1968a,b). It was shown in this paper that the lack of activity of pyrophosphorylases in extracts from *Shigella flexneri* is probably due to high activity of phosphatase in this species. Apart from the presence of this counteracting enzyme, all the pyrophosphorylases synthetizing ADPglucose, CDPglucose, GDPglucose, dTDPglucose and UDPglucose were detected in *Sh. flexneri*, similarly as in the other gram-negative bacteria studied so far, and the elution volume of a given enzyme on gel filtration was similar irrespective of the kind of bacteria.

In contrast to *S. typhimurium* and *E. coli* in which the composition of O-antigenic polysaccharide does imply the involvement of larger variety of nucleoside diphosphate sugar pyrophosphorylases, the composition of the O-antigenic polysaccharide in all types of *Shigella flexneri* suggests the demand for only those requiring UTP and dTTP. According to Simmons (1969) the repeating unit of O-antigen in *Sh. flexneri* 2a contains only *N*-acetylglucosamine and rhamnose. The pyrophosphorylases requiring CTP or GTP that may initiate the formation of 3,6-dideoxyhexoses and mannose, respectively (Robbins *et al.*, 1967) in *Sh. flexneri* could therefore be unnecessary. As shown by Sigal, Cattaneo & Segel (1964) all types of nucleoside diphosphate glucose can be used in the formation of bacterial glycogen. With respect to the biosynthesis of O-antigenic polysaccharide its composition is specified probably on a further biosynthetic stage, i.e. on the level of transglycosylases as implied by Mills & Smith (1965) and Risse, Lüderitz & Westphal (1967). The transglycosylation step is in fact a two-stage process, the transfer on the lipid carrier and the subsequent transfer on the growing sugar polymer (Robbins *et al.*, 1967).

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PIROFOSFORYLAZY NUKLEOZYDODWUFOSFOCUKRÓW U *SHIGELLA FLEXNERI* I *E. COLI*

Streszczenie

Stwierdzono obecność pirofosforylaz ADPglukozy, CDPglukozy, GDPglukozy, dTDPglukozy i UDPglukozy u szczepu *Shigella flexneri* 2a i przeprowadzono częściowe rozdzielenie tych enzymów drogą filtracji na kolumnie Sefadeks G-200. Podobny przebieg elucji tych samych enzymów pochodzących z ekstraktów *E. coli* świadczy o ich podobieństwie u obu badanych bakterii.

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CALCIUM BINDING BY TROPONIN

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Binding of calcium by troponin has been studied under various conditions, and two classes of binding sites were found. As the tightly bound calcium was considered the exchangeable calcium not removable by Dowex 50. Loosely bound calcium was determined on Sephadex G-25 column equilibrated with free Ca^{2+}.

The amount of tightly bound calcium is not influenced by changes in pH in the range between 6.5 and 8.0, by the presence of $MgCl_2$ or $SrCl_2$, by blocking of troponin SH groups, treatment of troponin with urea or heating to 100°C. The maximum number of tightly bound calcium extrapolated from Scatchard plot is about 4 moles per 10^5 g troponin, and the apparent binding constant greater than 10^6 M^{-1}. Corresponding values for loosely bound calcium are 11 moles per 10^5 g troponin and about 10^4 M^{-1}.

According to the generally accepted view calcium is a physiological regulator of the contraction-relaxation cycle. Ca^{2+} ions which are stored during relaxation inside the sarcoplasmic reticulum system are released after stimulation into sarcoplasma and activate contraction.

It is also well established that interaction of myosin with actin in the presence of ATP and Mg^{2+} represents an essential mechanism of muscle contraction and that minute amounts of Ca^{2+} are necessary to activate this interaction (for review see Ebashi & Endo, 1968a). Studies of Ebashi & Ebashi (1964) led to the discovery of a myofibrillar protein factor responsible for the so-called calcium sensitivity of actomyosin system. Only in the presence of this factor a relaxation of actomyosin system, coupled with the inhibition of the Mg^{2+}-stimulated ATPase, takes place when Ca^{2+} is removed either by sarcoplasmic reticulum or by Ca^{2+} chelators like EGTA[1]. Subsequently the calcium sensitizing factor was found to be a complex of tropomyosin and another protein called troponin (Ebashi & Kodama, 1965). Recent studies of Ebashi, Kodama & Ebashi (1968), Fuchs & Briggs (1968) and Drabikowski, Baryłko, Dąbrowska & Nowak (1968) showed that troponin contained

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[1] Abbreviations: EGTA, ethylene glycol bis-(β-aminoethylether)-N,N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PCMB, p-chloromercuribenzoate; NEM, N-ethylmaleimide.

tightly bound calcium and, consequently, Ebashi *et al.* (1968) as well as Fuchs & Briggs (1968) suggested that troponin is the Ca^{2+}-receptive protein in myofibrils and that its calcium-binding capacity is directly related to the sensitivity of the acto-myosin system to calcium ions.

The published data concerning the binding of calcium to troponin were, however, scarce and partially divergent. In the present work more detailed studies on the binding of calcium by troponin were performed in order to shed more light on the role of this protein in muscle.

MATERIAL AND METHODS

Preparation of troponin. Minced rabbit muscles were extracted twice for half an hour with 2 volumes of Guba-Straub solution, washed twice with 0.02 M-KCl - 3 mM-NaHCO$_3$, twice with water and, subsequently, extracted for 3 - 4 days with 1 mM-NaHCO$_3$ at 0° - 2°C. If necessary, pH of the suspension was adjusted to pH 8.0 with Na$_2$CO$_3$.

From the obtained extract a fraction precipitating between 0.4 and 0.6 ammonium sulphate saturation was collected which corresponded to tropomyosin-troponin complex. After removal of $(NH_4)_2SO_4$ by dialysis, KCl was added to 1 M concentration and pH of the solution was adjusted to 4.5 - 4.6. The precipitated tropomyosin was centrifuged off. To the supernatant adjusted to pH 7.0 ammonium sulphate was added to 0.7 saturation in order to remove nucleoprotein contaminations remaining under these conditions in the supernatant. The precipitate containing troponin was collected and, after dialysis against 1 mM-NaHCO$_3$, was used for further studies. Sometimes, instead of the last step of ammonium sulphate fractionation, pH 4.6 supernatant after neutralization and removal of KCl by dialysis was chromatographed on a Sephadex G-200 column. No difference was found between these two kinds of preparations. During all steps of the preparation 0.1 mM-β-mercaptoethanol was present. For details of the whole procedure see Drabikowski, Dąbrowska & Nowak (1969a). Before use troponin was treated with Dowex 50 to remove contaminations of free calcium.

Dowex 50 (W-X4 or W-X2, 200 - 400 mesh) was purified as described by Strzelecka-Gołaszewska & Drabikowski (1967) and used in tris form. To remove free calcium the solutions were gently shaken for 2 min with 1/7 volume of the Dowex 50 suspension (1:1) in 5 mM-tris-HCl, pH 7.5. The resin was removed by centrifugation.

Sephadex G-25 was washed with EDTA solution and then exhaustively with deionized water. Chromatography was performed on the columns 45 cm × 1 cm.

Radioactivity was measured in a Nuclear Chicago Corporation gas-flow counter with "Micromil" window or in a Packard liquid scintillation counter.

Protein concentration was determined with biuret reagent (Gornall, Bardawill & David, 1949) or according to Lowry, Rosebrough, Farr & Randall (1951).

Chemicals. Dowex 50 was purchased from Fluka A.G. (Buchs S. G., Switzerland), Sephadex G-25 from Pharmacia (Uppsala, Sweden), ATP disodium salt from Merck

(Darmstadt, West Germany), EGTA from Sigma Chemical Company (St. Louis, Mo., U.S.A.), EDTA from Ciech (Poland), PCMB and NEM from Fluka A. G. (Buchs S. G., Switzerland) and $^{45}CaCl_2$ from Biuro Dystrybucji Izotopów (Warszawa, Poland).

Deionized water was used throughout the whole procedure. Whenever possible, plastic vessels were used. Plastic and glassware were washed before use with EDTA and then exhaustively with deionized water.

RESULTS

Our previous studies (Drabikowski *et al.*, 1968) showed that troponin preparation contained about 5 moles of calcium not removable by Dowex 50 per 10^5 g protein and this calcium was considered as the tightly bound one. On the other hand, the amount of bound calcium calculated on the basis of isotopic dilution after exchange with $^{45}CaCl_2$ was found in general smaller, on the average about 2 moles per 10^5 g troponin. Those results suggested that only a part of the troponin-bound calcium was exchangeable. Previously the exchange experiments were performed only at 0.1 mM concentration of free $^{45}Ca^{2+}$ and preliminary results showed that the amount of exchangeable calcium depended to some extent on the concentration of free calcium. In that work the amount of bound exchangeable calcium (Ca_b) was calculated on the assumption that its specific activity after exchange $\left(\dfrac{a_b}{Ca_b}\right)$ was equal to the specific activity of the total calcium pool, i.e. calcium added (Ca_a) plus calcium bound (Ca_b).

$$\frac{a_b}{Ca_b} = \frac{a_a}{Ca_b + Ca_a} \qquad Ca_b = \frac{a_b \cdot Ca_a}{a_a - a_b} \qquad (1)$$

(a_b, bound radioactivity; a_a, added radioactivity).

One can see from Fig. 1 that in spite of some differences in the absolute values of the bound calcium obtained in various experiments, at the concentration of 0.1 mM of free $^{45}CaCl_2$ corresponding to about 6-8 moles of introduced calcium per 10^5 g protein, the amount of bound exchangeable calcium was usually 2.0-3.0 moles per 10^5 g in agreement with the previous results obtained under similar conditions. After incubation with much higher concentration of calcium the amount bound usually increased up to about 3.5 to 4.5 moles per 10^5 g protein. Equation 1 used for calculation of the amount of exchangeable bound calcium was based on the assumption that the amount of calcium bound to troponin (Ca_b) was the same before and after exchange. This could be not necessarily true, if troponin preparations were not always "saturated" with calcium. If the value of Ca_b before exchange was very small one can calculate the amounts of bound calcium directly on the basis of the specific activity of the added calcium $\left(\dfrac{a_a}{Ca_a}\right)$ with the use of the equation:

$$Ca_b = \frac{a_b \cdot Ca_a}{a_a} \qquad (2)$$

The obtained values of bound calcium although different from those calculated on the basis of equation (1) also show dependence on the concentration of added calcium. Regardless of the true value of Ca_b in initial troponin preparations at the higher ratios of Ca_a to Ca_b the possible differences in the values of Ca_b before and after exchange do not influence significantly the calculation and in fact the calculation with the use of equation (2) gives also the value of about 4 moles of Ca bound per 10^5 g troponin. Taking into account that the total amount of calcium not removable by Dowex 50 in troponin preparation was found before to be 4 - 5 moles per 10^5 g (Drabikowski et al., 1968) the present results suggest that almost all troponin-bound calcium is exchangeable.
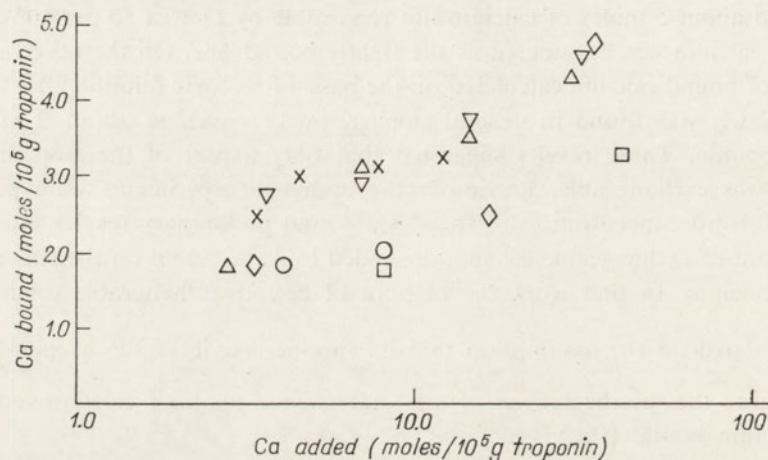

Fig. 1. Binding of exchangeable calcium by troponin. Troponin (2 - 3 mg/ml) in 4 mM-tris-HCl, pH 7.5, was incubated for 20 min with $^{45}CaCl_2$ at concentrations ranging from 0.05 to 1 mM. After subsequent treatment with Dowex 50 radioactivity was determined. Different symbols denote different experiments. In the series denoted by crosses, $^{45}CaCl_2$ was in all samples at 0.1 mM concentration, whereas concentration of troponin varied from 0.50 to 3.0 mg/ml.

It is at present difficult to explain satisfactorily the dependence of the amount of exchangeable bound calcium on the concentration of free calcium. The same pattern was obtained when for exchange-experiments troponin "saturated" with calcium was used. When troponin was incubated with 1 mM-$^{40}CaCl_2$ and treated with Dowex 50, the subsequent exchange with $^{45}CaCl_2$ led to the same amounts of incorporated radioactivity as in the control troponin not preincubated with $^{40}CaCl_2$. In all cases after exchange bound calcium was not in equilibrium with the free one, since the latter was completely removed with Dowex 50. On the other hand, at higher concentration of added calcium some additional binding of calcium not removable by short Dowex 50 treatment cannot be excluded. It is, however, worthwhile to mention that in similar exchange experiments with actin the amounts of bound calcium, calculated also on the basis of radioactivity remaining bound to G-actin after Dowex 50 treatment, were the same although the concentration of free $^{45}CaCl_2$ varied from 0.05 to 0.2 mM.

Table 1 shows the results of a "back" exchange, in an experiment in which troponin was first labelled with $^{45}CaCl_2$ and subsequently the exchange with free $^{40}Ca^{2+}$ was examined. In this case full 100% exchange of previously exchanged calcium was found at all concentrations of free calcium added.

Table 1

Exchange of ^{45}Ca bound to troponin with free $^{40}CaCl_2$

Troponin (2.5 mg/ml) after equilibration with $^{45}CaCl_2$ and subsequent treatment with Dowex 50 was incubated with various concentrations of $^{40}CaCl_2$ for 20 min. After the next Dowex 50 treatment radioactivity was measured. Per cent of exchange was calculated according to the following equation:

$$\frac{(a_0 - a_b)\,(Ca_b + Ca_i)}{a_0 \cdot Ca_i} \cdot 100$$

a_0, radioactivity bound before exchange; a_b, radioactivity bound after exchange; Ca_b, concentration of bound calcium; Ca_i, concentration of free calcium.

$^{40}CaCl_2$ added, (mM)	^{45}Ca bound after exchange (counts/min/mg protein)	% of exchange
None (control)	8400	—
0.05	3990	101.0
0.1	2530	104.0
0.2	2100	95.0
0.5	1000	97.4

The rate of exchange of troponin-bound calcium with free calcium was very fast (after two minutes the exchange was already complete) and therefore it was too difficult to study its kinetics.

In the pH range from 6.5 to 8.0 no difference in the amount of exchangeable calcium remaining bound after Dowex 50 treatment was found.

The presence of tropomyosin, or serum albumin used for comparison, had no effect on the amount of radioactivity incorporated during the exchange (Table 2).

Both $MgCl_2$ and $SrCl_2$ present during the incubation of troponin with $^{45}CaCl_2$, had only a slight effect on the exchange (Table 3). Similarly, incubation with $MgCl_2$ or $SrCl_2$ of troponin labelled with ^{45}Ca and freed of free Ca^{2+} caused only a small release of bound radioactivity as found after subsequent Dowex 50 treatment. All these experiments indicate the lack of significant replacement of troponin-bound calcium by these cations.

The presence of 1 - 2 mM-ATP caused some 10 - 15% decrease of the amount of calcium bound to troponin after Dowex 50 treatment. Control experiments showed that this decrease could be only partially attributed to the contaminations of calcium present in ATP preparations.

Blocking of troponin sulphydryl groups by NEM was without any effect and that by PCMB had only very slight effect on the incorporation of radioactive Ca^{2+}

Table 2

The effect of tropomyosin and serum albumin on the binding of calcium by troponin

Troponin was equilibrated with 0.1 mM-^{45}CaCl$_2$ in the absence or in the presence of various amounts of tropomyosin or serum albumin, and after treatment of Dowex 50 radioactivity was determined.

Expt. no.	Protein (mg/ml)			^{45}Ca bound (counts/min/mg troponin)
	troponin	tropomyosin	albumin	
1	1.36	—	—	10400
	1.36	0.40	—	10400
	1.36	—	0.50	10700
2	1.02	0.93	—	11300
	1.02	—	0.82	10250
3	0.69	1.08	—	10100
	0.69	—	1.09	10700
	—	1.38	—	136
	—	—	1.22	0

Table 3

The effect of MgCl$_2$ *and* SrCl$_2$ *on the exchange of troponin-bound calcium with* ^{45}CaCl$_2$

Samples of troponin were incubated for 20 min with 0.1 mM-^{45}CaCl$_2$ alone or in the presence of other cations, as indicated in the Table. After subsequent Dowex 50 treatment radioactivity was measured.

Expt. no.	Addition	^{45}Ca bound after exchange (counts/min/mg troponin)
1	None	4600
	0.1 mM-MgCl$_2$	3800
	0.1 mM-SrCl$_2$	3660
	0.1 mM-^{40}CaCl$_2$	2570
2	None	6800
	0.1 mM-MgCl$_2$	6200
	0.1 mM-SrCl$_2$	6420
	0.1 mM-^{40}CaCl$_2$	4340
3	None	7750
	0.1 mM-MgCl$_2$	7230
	0.2 mM-MgCl$_2$	7420
	1.0 mM-MgCl$_2$	6820

into troponin (Table 4). Similarly, incubation of troponin for 20 hours with 6 M-urea, followed by its removal by dialysis, had no influence on the amount of bound exchangeable calcium.

Heat denaturation did not affect the calcium binding ability of troponin. During 5 min incubation at 100°C troponin partially precipitated giving two fractions of different composition, as judged by polyacrylamide disc electrophoresis (in pre-

Table 4

Effect of NEM and PCMB on the binding of exchangeable calcium by troponin

Samples of troponin were incubated at 0°C, for 24 h with NEM or PCMB at the concentrations indicated, then for 20 min with 0.1 mM-^{45}CaCl$_2$. After subsequent treatment with Dowex 50 radioactivity and protein content were measured.

Expt. no.	Addition	^{45}Ca bound (counts/min/mg troponin)
1	None, control	4950
	NEM, 28 mM	5020
	PCMB, 1.5 mM	4500
2	None, control	3770
	NEM, 1 mM	3980
	NEM, 28 mM	3670
	PCMB, 1 mM	3260

paration). Most of originally bound calcium remained bound to the soluble fraction.

The determination of bound calcium after complete removal of free calcium by treatment with Dowex 50 did not enable to estimate the binding constant. For this a resin partition method similar to that previously used by Fuchs & Briggs (1968) was employed, in which Dowex 50 was used instead of Chelex 100. The results are presented in Fig. 2 in the form of a Scatchard (1949) plot. Although each series of experiments led to somewhat different results which made rather difficult to plot the curve, it can be seen that the presence of 2 mM-SrCl$_2$ or MgCl$_2$ had no effect on the binding of calcium. The plot was always not linear indicating that there were at least two classes of calcium-binding sites in troponin. Hence, the values extrapolated from the Scatchard (1949) plot can be accepted only as the approximate ones. For the class of tighter binding sites the extrapolated maximum number of binding sites seems to be about 4 moles per 10^5 g troponin and the value for the apparent affinity constant at least of the order of 10^6 M^{-1}. That the affinity of calcium to troponin can be so high was indicated by the results of experiments on the effect of calcium chelators, EDTA and EGTA, on the binding of calcium by troponin. In these experiments zonal chromatography on Sephadex G-25 column was adapted for the separation of calcium released from troponin by the chelators from that remaining bound (Fig. 3). Ion exchangers could not be used for this purpose since control experiments showed that, contrary to the observations of Maruyama (1962), neither Dowex 1 nor Dowex 50 removed all EDTA-^{45}Ca complex from the protein-free solution. When samples of troponin equilibrated with 0.1 mM-^{45}CaCl$_2$ but not treated with EDTA or EGTA were passed through a Sephadex G-25 column the peak corresponding to free calcium ions was not observed in the effluent, due to absorption of calcium ions on Sephadex. Apart from this fact, the amount of radioactive calcium found after chromatography in the protein-containing peak was very similar to that remaining bound to troponin after Dowex 50 treatment

(Table 5). As reported recently by Drabikowski, Baryłko, Dąbrowska & Sarzała (1970) in the presence of 1 mM-EDTA or EGTA, i.e. when the concentration of free calcium was about 10^{-8} M, only a small part of calcium bound to troponin was removed. This amount was not influenced by the presence of 2 mM-MgCl$_2$. Somewhat higher release of bound Ca was found when 2 mM-ATP was present during incubation with EGTA and MgCl$_2$.
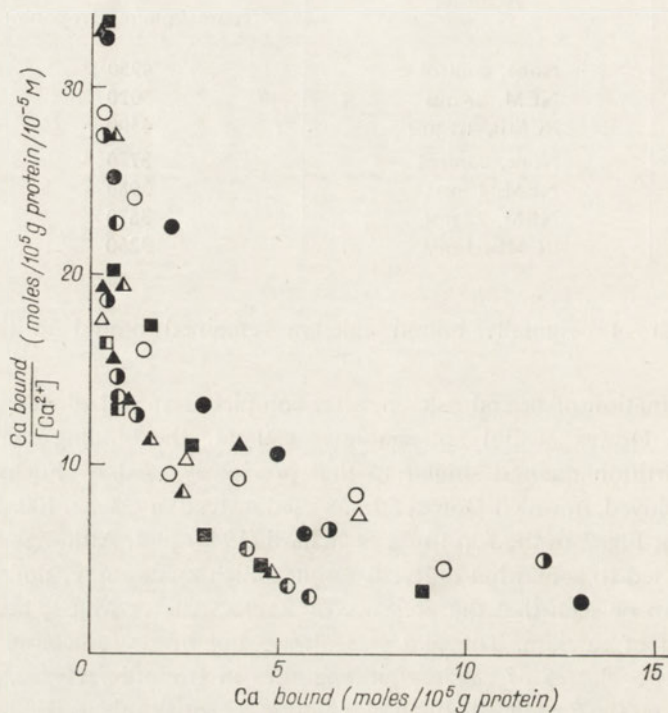

Fig. 2. Scatchard plot of calcium binding by troponin, assayed by the resin partition method. Troponin (0.12 - 0.16 mg/ml) in 60 mM-KCl - 30 mM-imidazole, pH 7.0, was incubated with various concentration of ^{45}CaCl$_2$ (a) in the absence of other bivalent cations (circles), (b) in the presence of 2 mM-MgCl$_2$ (triangles), or (c) in the presence of 2 mM-SrCl$_2$ (squares). After 20 min the mixture was treated with Dowex 50 which was preequilibrated with the KCl-imidazole solution, containing in addition 2 mM-MgCl$_2$ in (b) and 2 mM-SrCl$_2$ in (c). After removal of resin, radioactivity was determined. In the parallel series in which troponin was omitted the amount of calcium not removed by resin was determined. For calculation of bound exchangeable calcium the equations of Fuchs & Briggs (1968) were used. On the figure the results of four experiments are presented.

Determination of binding constant using resin partition method described above suggested the presence in troponin of separate class of more loosely bound calcium. To obtain more information about this class of calcium-binding sites, the method of Hummel & Dreyer (1962) was used based on the determination of the amount of ligand bound to protein on Sephadex columns equilibrated with free ligand. Figure 4 shows an elution profile of troponin from a Sephadex G-25
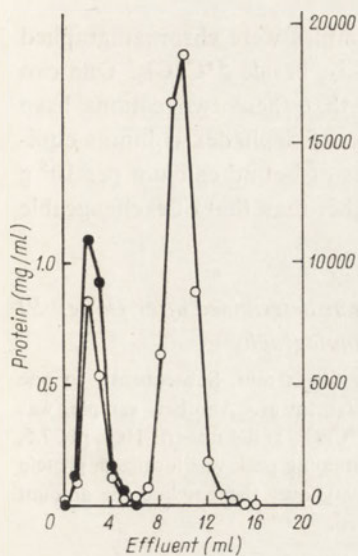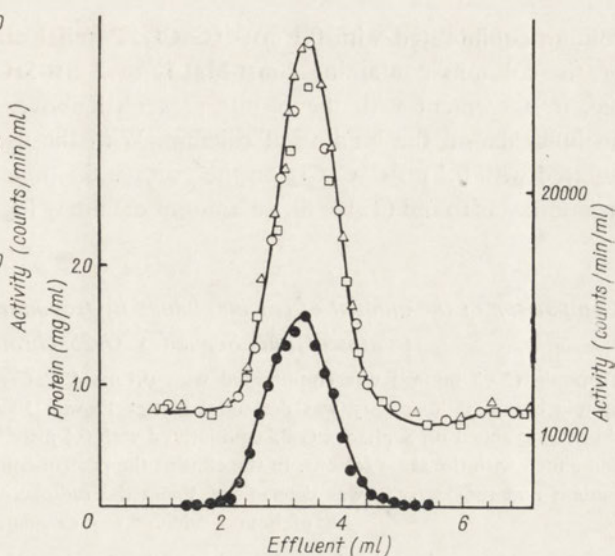
Fig. 3. Zonal chromatography of troponin-^{45}Ca on Sephadex G-25 column after incubation with EDTA. Troponin solution, 1.5 ml, after equilibration for 20 min with 0.1 mM-^{45}CaCl$_2$ was incubated for one hour at 0°C with 1 mM-EDTA and then passed through Sephadex G-25 column (45 × 1 cm) with 4 mM-tris-HCl, pH 7.5, as eluent. One ml fractions were collected and radioactivity and protein content were determined. Sephadex columns before use were washed carefully with 0.1 mM-EDTA, water, and finally with 4 mM-tris-HCl, pH 7.5. ●, Protein content; ○, radioactivity.

Fig. 4. Determination of the binding of calcium by troponin with the equilibrated Sephadex G-25 column. 1.5 ml troponin (2 - 3 mg/ml) was applied to a Sephadex G-25 column (1 × 45 cm), ○, equilibrated with 0.1 mM-^{45}CaCl$_2$ - 4 mM-tris-HCl, pH 7.5, and eluted with the same solution. In other columns, □, 2 mM-MgCl$_2$ or △, 2 mM-SrCl$_2$ were additionally present. In the effluents ○, □, △, radioactivity and ●, protein concentration were determined. The amount of bound calcium was calculated directly from the number of counts per one mg protein present in the protein peak above the baseline and specific activity of used ^{45}CaCl$_2$. On the figure only the first part of elution profile containing the protein peak is presented.

Table 5

Effect of EGTA on the binding of exchangeable calcium by troponin

Samples of troponin after equilibration with 0.1 mM-^{45}CaCl$_2$ were incubated at 0°C for 1 h under conditions as indicated in the Table. After incubation calcium not bound to troponin was separated on the Sephadex G-25 column as described in legend to Fig. 3. In the control samples of the initial troponin solutions the amount of bound radioactivity was determined after Dowex 50 treatment.

Expt. no.	Dowex 50 treated (control)	^{45}Ca bound (counts/min/mg protein)			
		After separation on Sephadex column			
		Samples incubated with the addition of			
		None	1 mM-EGTA	1 mM-EGTA, 2 mM-MgCl$_2$	1 mM-EGTA, 2 mM-ATP, 2 mM-MgCl$_2$
1	5300	5520	5200	—	—
2	5380	5260	3770	—	2660
3	2920	2890	2170	2050	1630

column equilibrated with 0.1 mM-^{45}CaCl$_2$. Parallel samples were chromatographed on the columns containing 2 mM-MgCl$_2$ or 2 mM-SrCl$_2$, beside ^{45}CaCl$_2$. One can see, in agreement with the results presented above, that these two cations have no influence on the binding of calcium. With the use of Sephadex columns equilibrated with 0.1 mM-^{45}CaCl$_2$ on the average 6.5 moles of bound calcium per 10^5 g troponin was found (Table 6), an amount definitely higher than that of exchangeable

Table 6

Comparison of the amount of calcium bound by troponin as determined after Dowex 50 treatment and Sephadex G-25 chromatography

Troponin (2 - 3 mg/ml) was equilibrated with 0.1 mM-^{45}CaCl$_2$ for 20 min. Subsequently in one sample bound radioactivity was determined after Dowex 50 treatment. Another sample was chromatographed on Sephadex G-25 equilibrated with 0.1 mM-^{45}CaCl$_2$ and 4 mM-tris-HCl, pH 7.5, and eluted with the same solvent. In the effluent the protein-containing peak was localized, protein content and radioactivity was determined. From the radioactivity over the baseline the amount of bound calcium was calculated.

Expt. no.	^{45}Ca bound (moles per 10^5 g troponin)	
	after Sephadex G-25 column chromatography	after Dowex 50 treatment
1	6.0	2.3
2	4.9	1.7
3	6.2	2.1
4	7.7	1.4
5	7.0	2.2
6	7.1	3.2
Average	6.5	2.2

calcium measured after Dowex 50 treatment. Treatment with Dowex 50 of that portion of the effluent from Sephadex column in which protein was detected, decreased the amount of bound calcium to the same value as that found in the original troponin after Dowex 50 treatment. This observation furnished additional evidence that the method of Hummel & Dreyer (1962) enabled detection of calcium loosely bound to troponin which is removable by Dowex 50.

Scatchard plot of the values obtained in the experiments in which troponin was chromatographed on Sephadex columns equilibrated with various concentrations of ^{45}CaCl$_2$ (Fig. 5) clearly shows the complex character of calcium binding. The maximum extrapolated value of bound calcium was found to be about 19 moles per 10^5 g troponin. From this amount about 11 moles seem to belong to the class of loosely bound calcium. The calculated value of affinity constant for this class of binding sites is about 1.3×10^4 M^{-1}. Similar value can be obtained from the extrapolation of the lowest part of the curve from Fig. 2. The relation between the class of tighter binding sites seen on the curve in Fig. 5, for which the extrapolated value of maximum number of moles of bound calcium seems to be about 8 per 10^5 g protein and the class of binding sites for tightly bound calcium obtained in the experiments with the resin partition method (Fig. 2) remains to be elucidated.
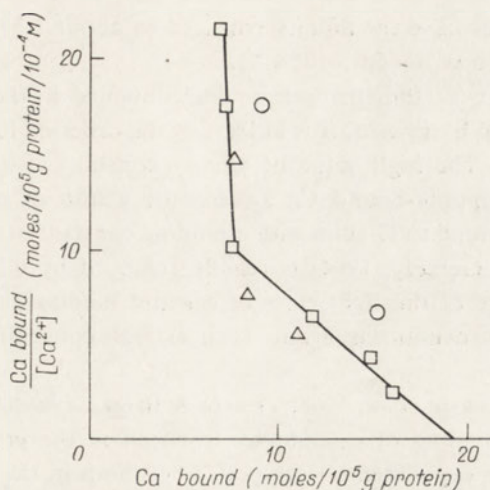
Fig. 5. Scatchard plot of the binding of calcium by troponin, assayed by equilibrated Sephadex G-25 column method. Samples of troponin after incubation with $^{45}CaCl_2$ at the concentrations ranging from 0.03 to 1 mM were chromatographed on Sephadex G-25 columns equilibrated with the same concentrations of $^{45}CaCl_2$ and 4 mM-tris-HCl, pH 7.5. Details of procedure as described in the legend to Fig. 4. On the figure the results of three experiments are presented.

DISCUSSION

The strong affinity of calcium to troponin was shown independently by Ebashi *et al.* (1968), Fuchs & Briggs (1968), Drabikowski *et al.* (1968) and Arai & Watanabe (1968), but the results obtained differed in respect to the amount of calcium bound, its exchangeability with free calcium and the value of binding constant. Ebashi and coworkers (1968b) found about 4.0 moles of bound Ca per 10^5 g troponin, whereas Fuchs & Briggs (1968) and Arai & Watanabe (1968) obtained only 2.2 moles per 10^5 g and 3.0 moles per 10^5 g, respectively, as the maximum extrapolated value from Scatchard plot. Both Ebashi *et al.* (1968b) and Fuchs & Briggs (1968) came to the conclusion that most of the bound calcium is exchangeable. On the other hand, we found by chemical determination about 5 moles of bound Ca per 10^5 g troponin but only about 2.0 moles of Ca per 10^5 g troponin when calculated on the basis of exchange with radioactive calcium (Drabikowski *et al.*, 1968). The results of this work based also on the short treatment with Dowex 50 seem to support the view that almost all calcium bound to troponin is exchangeable.

The results of the present work clearly indicate the existence of at least two classes of calcium-binding sites in troponin. As the maximum amount of tightly bound calcium extrapolated from Scatchard plot a value of about 4 moles Ca per 10^5 g troponin was obtained. This amount is rather higher than found by other workers, except that reported by Ebashi *et al.* (1968b).

The apparent affinity constant of calcium to troponin reported by other workers varied from 3.7×10^5 M^{-1} to 2.2×10^6 M^{-1}. Ebashi and coworkers (1968b) came to the conclusion that the obtained data can be better explained if instead of one class of binding sites with an affinity constant equal to 2.5×10^5 M^{-1} one assumes

that half of binding sites have the affinity constant of about 1.3×10^6 M^{-1} and the other half a constant equal to 5.0×10^4 M^{-1}.

The affinity constant for the stronger binding obtained in the present work is similar to that calculated by Ebashi; it is at least of the order of 10^6 M^{-1} or perhaps even somewhat higher. The high value of affinity constant also results from the strong resistance of troponin-bound Ca against the action of calcium chelators. For instance, calcium bound to G-actin with a binding constant of about 2×10^5 M^{-1} (Martonosi, Molino & Gergely, 1964) is readily removed by 0.2 mM-EDTA. The most unexpected feature of the tight class of calcium binding sites to troponin is its unusual resistance to denaturing agents such as high concentrations of urea or heating at 100°C.

All other workers (Ebashi *et al.*, 1968b; Fuchs & Briggs, 1968; Arai & Watanabe, 1968) determined the binding of calcium by troponin in the presence of MgCl$_2$. In the present work the experiments were performed both in the absence and presence of MgCl$_2$, hence, enabled to show that this cation had no effect on either class of calcium-binding sites. A similar observation was recently reported for the tight binding sites by Fuchs, Reddy & Briggs (1970). Also strontium, the affinity of which to cardiac troponin was according to Ebashi *et al.* (1968b) higher than that of calcium, has no effect on the binding of calcium by skeletal muscle troponin.

Recent studies indicate that troponin is in fact a complex of more than one protein. Hartshorne & Mueller (1968) were able to separate troponin into two fractions from which only one was subsequently found to contain most of the tightly bound calcium (Drabikowski, Dąbrowska, Baryłko, Greaser & Gergely, 1969b). Recently Greaser & Gergely (1970) and Drabikowski, Dąbrowska & Baryłko (1971) showed that among four protein fractions obtained from troponin on DEAE-Sephadex column only one — the most retarded fraction on DEAE-Sephadex column — appears to contain all tightly bound calcium of the initial troponin. In view of these observations the calculation of maximum number of moles of bound calcium per unit weight of the troponin complex has a very limited meaning and it was presented in this paper only in order to compare our results with those of other authors, which were also obtained for the unfractionated troponin. Our preliminary experiments seem to indicate that the other protein fractions present in troponin preparations have no effect on the calcium-binding ability of the particular fraction, at least for tighter binding sites. For instance the value of affinity constant for this class of binding sites determined in this work for the unfractionated troponin is very similar to the value estimated by Greaser & Gergely (1970) for the isolated calcium-binding fraction.

The existence of the second class of binding sites assumed by Ebashi has been evidently demonstrated in this work using a method of determination of binding on Sephadex columns equilibrated with Ca^{2+}. The maximum number of moles of this class of weakly bound calcium was found to be about 11 per 10^5 g troponin and the affinity constant of the order of 10^4 M^{-1}. Our knowledge about this class of binding sites is at present rather poor. Since the loosely bound calcium is removed by Dowex 50 one may suppose that it is similar to the loosely bound calcium in

actin studied by Martonosi *et al.* (1964). Besides, nothing is known so far which of the troponin fractions contains binding sites for loosely bound calcium.

The details of fractionation of troponin and studies on the properties of its constituents will be published elsewhere. One should mention, however, that we still use the name "troponin" for the complex of several components showing one specific property: inhibition of Mg^{2+}-stimulated ATPase activity of actomyosin in the presence of tropomyosin. However, if only one of the protein constituents of troponin binds calcium, small differences in the preparation procedure introduced by individual workers may change the proportions of the constituents in the troponin preparations and, consequently, may explain the differences in the amounts of bound calcium reported in the literature.

On the basis of high affinity toward calcium it has been postulated (Ebashi *et al.*, 1968a,b; Fuchs & Briggs, 1968) that troponin is the Ca^{2+}-receptive protein in myofibrils. This hypothesis has been recently called in question, among others on the basis of the observations that the binding of calcium to the tighter binding sites seems to be too strong to be directly involved in the contraction-relaxation cycle (Drabikowski *et al.*, 1970). On the other hand, the second class of binding sites has too low affinity to be involved directly in the contraction-relaxation cycle. Clearly much more work is needed to elucidate this problem. It is, however, worth to mention recent reports which seem to indicate some conformational changes in troponin as a whole, depending on the concentration of free calcium ions. On the basis of ultracentrifugation and polyacrylamide disc electrophoresis Wakabayashi & Ebashi (1968) suggested an aggregation of troponin at low Ca^{2+} concentrations. Other authors (Schaub & Perry, 1969; Chowrashi & Kaldor, 1970; Drabikowski *et al.*, 1970) also observed changes in the electrophoretic mobility of troponin or some of its subfractions, depending on the concentrations of calcium, but the results were not always consistent. The main difficulty concerned in this case the contradiction between the authors as to the number of bands revealed in troponin preparations in polyacrylamide disc electrophoresis. Tonomura, Watanabe & Morales (1969) employing "spin label" technique concluded that the interaction between F-actin and myosin is influenced by calcium-induced conformational changes in the tropomyosin-troponin system, and Han & Benson (1970) found that the addition of $1 - 2 \times 10^{-4}$ M-Ca^{2+} caused an increase of fluorescence intensity of troponin which also suggested some conformational changes induced by calcium. In the last case, in view of the results of this work, certainly the sites of loosely bound calcium become occupied. In view of the established heterogeneity of troponin preparations more work has to be performed on the isolated troponin fractions in order to show which of the troponin constituents is responsible for the phenomena observed.

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WIĄZANIE WAPNIA PRZEZ TROPONINĘ

Streszczenie

Badano wiązanie wapnia przez troponinę w różnych warunkach i znaleziono dwie klasy miejsc wiązania. Jako wapń silnie związany przyjęto wymienialny wapń, nieusuwany przez Dowex 50. Słabo wiązany wapń oznaczano na kolumnach z Sephadex G-25 zrównoważonych wolnym Ca^{2+}.

Na ilość wapnia silnie związanego nie ma wpływu pH w zakresie 6.5 - 8.0, obecność $MgCl_2$ lub $SrCl_2$, blokowanie grup SH troponiny, traktowanie troponiny mocznikiem oraz ogrzewanie do 100°C. Maksymalna ilość silnie związanego wapnia, ekstrapolowana z krzywej Scatcharda, wynosi około 4 mole na 10^5 g troponiny, a stała wiązania jest większa od 10^6 M^{-1}. Wartości dla luźno związanego wapnia wynoszą odpowiednio 11 moli na 10^5 g troponiny i 10^4 M^{-1}.

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COMPUTER STATISTICAL ANALYSIS OF THE MICHAELIS CONSTANT ESTIMATIONS

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1. Michaelis constants for AMP-aminohydrolase purified from hen, chicken and carp muscles have been calculated, using computer statistical analysis, by three different linear transformations of the Michaelis-Menten equation. 2. On analysing the results of 110 experiments, it has been observed that the most frequently used double reciprocal plot: $\frac{1}{v} = f\left(\frac{1}{S}\right)$ fits reasonably well both to good and poor experimental data. The linear transformation most efficiently discriminating poor experimental data in graphical determination of K_m, seems to be that expressed by the $v = f\left(\frac{v}{S}\right)$ function.
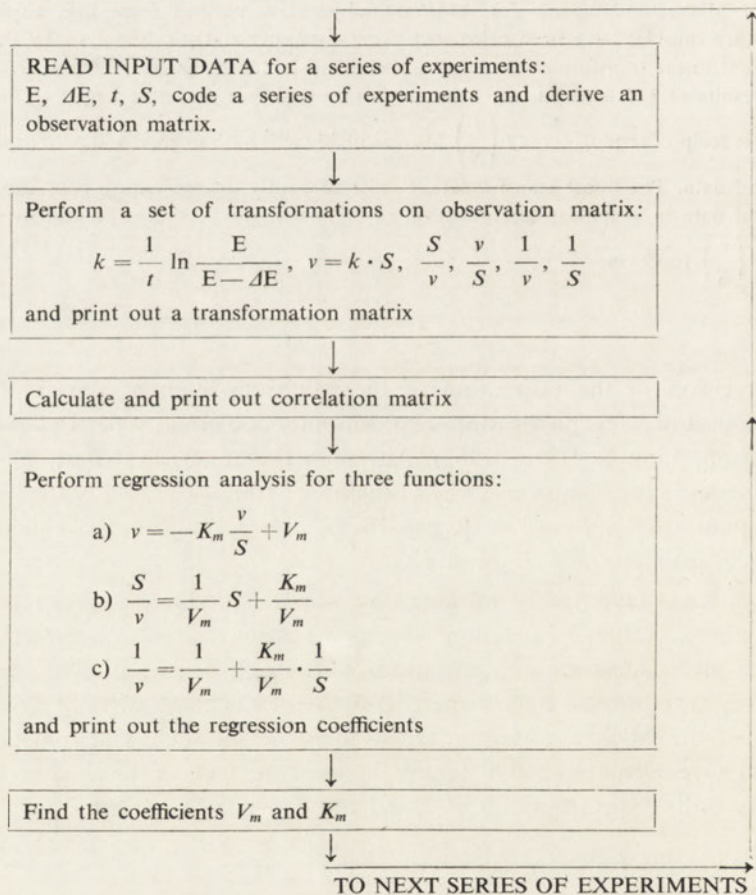
The precision of the estimation of the Michaelis-Menten constant (K_m) has been the subject of a few publications (e.g. Mounter & Turner, 1963; Dowd & Riggs, 1965; Hanson, Ling & Havir, 1967). Although the Lineweaver-Burk plot of the enzyme kinetics is the less precise way of the linear transformation of the Michaelis-Menten equation (e.g. Dowd & Riggs, 1965), it is astonishingly frequently used for graphical estimation of K_m and V_{max}.

Dowd & Riggs (1965) were working on computer-produced figures representing randomly chosen values of reaction velocity v scattered around a theoretical true value for a given substrate concentration S. The aim of the present paper is to revaluate on experimental figures the usefulness of the three ways of linear transformation of the Michaelis-Menten equation for graphical calculation of the K_m value. Data have been obtained by taking the measurements of the reaction velocities on a self-recording spectrophotometer and the data were rearranged on ICT digital computer as the following functions: $v = f_1\left(\frac{v}{S}\right)$, $\frac{S}{v} = f_2(S)$ and $\frac{1}{v} = f_3\left(\frac{1}{S}\right)$.

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MATERIALS AND METHODS

AMP-aminohydrolase has been purified from hen and chicken muscles as described by Kaletha & Żydowo (1971), and from carp muscle according to Purzycka & Żydowo (1969). The reaction rates were measured by recording the decrease in extinction at 265 nm on a self-recording Unicam SP-800 spectrophotometer, fitted with a constant-temperature cell-housing; the decrease in extinction at this wavelength is due to the difference in extinction coefficients of AMP and IMP. The reaction mixture contained 500 mM-KCl, 50 mM-K-succinate buffer, pH 6.6, and varying concentrations of AMP in a final volume of 3 ml. The reaction was started by the addition of 20 μl of appropriately diluted enzyme solutions and carried out for 6 min while recording the extinction continuously. The recorded reaction was of the first kinetic order, therefore the reaction rate constant k was calculated and its product by initial substrate concentration S was taken as velocity. Initial substrate concentration was checked by measuring the extinction at 265 nm.

The measurements have been used as input data for a program "STATISTICAL ANALYSIS ICL" = XDS2 (Statistical analysis MARK 2, London 1969) on ICT-1904 digital computer, according to the following algorithm:

READ INPUT DATA for a series of experiments:
E, ΔE, t, S, code a series of experiments and derive an observation matrix.

Perform a set of transformations on observation matrix:
$$k = \frac{1}{t} \ln \frac{E}{E - \Delta E}, \quad v = k \cdot S, \quad \frac{S}{v}, \quad \frac{v}{S}, \quad \frac{1}{v}, \quad \frac{1}{S}$$
and print out a transformation matrix

Calculate and print out correlation matrix

Perform regression analysis for three functions:

a) $v = -K_m \dfrac{v}{S} + V_m$

b) $\dfrac{S}{v} = \dfrac{1}{V_m} S + \dfrac{K_m}{V_m}$

c) $\dfrac{1}{v} = \dfrac{1}{V_m} + \dfrac{K_m}{V_m} \cdot \dfrac{1}{S}$

and print out the regression coefficients

Find the coefficients V_m and K_m

TO NEXT SERIES OF EXPERIMENTS

Materials. AMP was a Fluka (Buchs, Switzerland) product, all the other chemicals were purchased from Centrala Odczynników Chemicznych (Gliwice, Poland). Hens were purchased from Przedsiębiorstwo Jajczarsko-Drobiarskie in Gdańsk, fertilized hen eggs from Przedsiębiorstwo Wylęgu Drobiu in Pruszcz Gdański, and carps from Centrala Rybna in Gdańsk. Ammonia-free glass-distilled water was used throughout the experiments.

RESULTS AND DISCUSSION

As it may be seen from Table 1, the K_m values obtained from the same experimental data may differ considerably depending on the method of linear transformation of the equation; this is not a result of graphical error as the values have been obtained numerically with the same precision. The values of K_m calculated from the same experimental data differed by as much as two orders of magnitude (e.g. experiment no. 5 in Table 1). The most varied values of K_m were obtained while using the Lineweaver-Burk plot $\left[\frac{1}{v}=f\left(\frac{1}{S}\right)\right]$, while the linear transformation represented by the function $v=f\left(\frac{v}{S}\right)$ resulted in the smallest scattering of the K_m values in a given group of experimental data. The question marks in Table 1 refer to the cases in which computer

Table 1

K_m *values of AMP for AMP-aminohydrolase, obtained by three different linear transformations* ($\times 10^{-3}$M) *of the Michaelis-Menten equation*

No. of measurements in replicates	$v=f_1\left(\frac{v}{S}\right)$	$\frac{S}{v}=f_2(S)$	$\frac{1}{v}=f_3\left(\frac{1}{S}\right)$
The enzyme from 1 day-old chicken (30°C)			
15	2.9	3.8	3.5
16	3.7	4.8	4.8
31	6.8	15.6	82.9
1	?	?	21.9
49	3.1	3.9	7.6
The enzyme from adult hen (40°C)			
112	?	1.6	14.4
114	3.9	6.6	9.3
22	7.0	10.9	41.6
5	3.8	22.7	335.0
77	10.9	14.5	21.0
The enzyme from carp (35°C)			
127	1.2	1.2	2.0
129	1.7	2.0	2.5
130	3.6	4.0	6.2
128	1.5	1.9	1.1
98	0.8	?	2.0
97	?	?	0.5

gave no answer because a nonsense value of K_m (e.g. negative value) resulted from the unavoidable experimental errors. The lack of answer occurred with all the methods of linear transformation although it did not depend on distribution of the input data around the straight line, as may be seen from Table 2. It is remarkable that correlation coefficient r for the fitting of data to the function $\frac{1}{v} = f\left(\frac{1}{S}\right)$ was always high and never decreased below 0.5. The same data rearranged as the functions $v = f\left(\frac{v}{S}\right)$ or $\frac{S}{v} = f(S)$ resulted in several cases in lower correlation coefficients. In 43 cases out of 110 the correlation coefficient was lower than 0.5 while using $v = f\left(\frac{v}{S}\right)$ function for calculation (Table 2).

Table 2

Comparison of results obtained by three methods of K_m estimation

Total number of experiments: 110; r, correlation coefficient.

	$v = f_1\left(\dfrac{v}{S}\right)$	$\dfrac{S}{v} = f_2(S)$	$\dfrac{1}{v} = f_3\left(\dfrac{1}{S}\right)$
Number of nonsense results	23	40	30
Number of results with $r < 0.5$	43	25	0

The presented results indicate that the Lineweaver-Burk plot is the most convenient but unprecise way of linear transformation of Michaelis-Menten equation. Both good and poor experimental data fit within reasonably small scatter, but the K_m values obtained in this way are subject to large error. This is in agreement with the results of Dowd & Riggs (1965), who recommended the $v = f\left(\frac{v}{S}\right)$ function as the most suitable and precise way of K_m estimation. Taking this into account a program in FORTRAN has been set up permitting the K_m and V_m estimates (Hanson et al., 1967). However, an ALGOL program has been published recently based still on the $\frac{1}{v} = f\left(\frac{1}{S}\right)$ linear transformation (Hay & Goldberg, 1971). One can hardly find any enzymological publication in which the kinetic data would be presented in a way other than the double reciprocal plot according to Lineweaver and Burk. The results presented here confirm the opinion of Dowd & Riggs (1965) that the popularity of the Lineweaver-Burk method is based upon the ability to provide what seems to be a good fit even when the experimental data are poor.

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KOMPUTEROWA ANALIZA STATYSTYCZNA WYZNACZANIA STAŁEJ MICHAELISA

Streszczenie

1. Obliczono stałe Michaelisa dla AMP-aminohydrolazy oczyszczonej z mięśni kury, kurczęcia i karpia, posługując się komputerową analizą statystyczną różnych zależności liniowych opartych na równaniu Michaelisa-Mentena.

2. Analizując 110 doświadczeń zaobserwowano, że najczęściej stosowana metoda Lineweavera-Burka: $\frac{1}{v} = f\left(\frac{1}{S}\right)$ nie pozwala na zróżnicowanie prawidłowych danych doświadczalnych od danych obarczonych dużym błędem. Sposobem najskuteczniej dyskryminującym błędne dane pomiarowe przy graficznym wyliczaniu K_m, jest zależność wyrażona funkcją: $v = f\left(\frac{v}{S}\right)$.

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INTERACTION OF *ESCHERICHIA COLI* RIBOSOMES WITH NATURAL AND SYNTHETIC TEMPLATES

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1. Monovalent cations replace to a large extent magnesium in the interaction of *E. coli* mRNA with ribosomes, whereas in the case of poly U divalent cations appeared to be essential. 2. The *E. coli* mRNA-ribosome complex in the presence of both Mg^{2+} and K^+ dissociates at an urea concentration as high as 4 M, whereas in the presence of only one of them, at 1 M-urea. On the other hand, the poly U-ribosome complex in the presence of Mg^{2+} alone is resistant to 1 M-urea. 3. The *E. coli* mRNA-ribosome complex in the presence of Mg^{2+} is stable over the pH range 5 to 9.5. 4. Formylation of ribosomes results in the loss of their ability to bind either poly U or natural templates. 5. Blocking of the amino groups of ribosomal protein with DNFB impairs the interaction of ribosomes with natural templates without having a significant effect on binding of poly U.

The mechanism of binding of messenger RNA (mRNA) to ribosomes is still to be elucidated. There are two hypotheses. One is based on the experiments which demonstrated the involvement of magnesium ions in binding of natural and synthetic mRNA's to ribosomes (Gros *et al.*, 1961; Spirydes & Lipmann, 1962). This hypothesis (Watson, 1964) assumed that mRNA is bound to ribosomes through magnesium bridges between phosphate residues of mRNA and of ribosomal RNA (rRNA). According to the second hypothesis (Moore, 1966a,b), mRNA is bound to ribosomes by hydrogen bonds between amino groups of rRNA and phosphate of mRNA, magnesium being only indirectly involved. This model of attachment is based on the results of experiments on binding of polyuridylic acid (poly U) to *E. coli* ribosomes. However, it is not certain whether this is also the case with the attachment of natural mRNA's to ribosomes, as translation of natural mRNA is dependent on initiation factors whereas the synthesis of polyphenylalanine is not (Eisenstadt & Brawerman, 1966; Revel & Gros, 1966; Stanley, Salas, Wahba & Ochoa, 1966; Revel, Herzberg, Becarevic & Gros, 1968).

In the present work, the mechanism of *E. coli* mRNA, phage f2 RNA, as well as poly U binding to *E. coli* ribosomes, was studied. Two aspects were taken into

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account: *a)* the role of cations in the stability of ribosome-mRNA complex, and *b)* the role of ribosome structure in binding of mRNA. The results indicate that there are some differences in the mechanisms involved in the interaction of ribosomes with poly U and with natural mRNA's. The binding of the natural mRNA's requires the participation of amino groups of ribosomal proteins which are not essential for binding of poly U.

MATERIALS AND METHODS

Reagents. These were from the following sources: [^{14}C]adenine, sp. act. 35 Ci/mol, from the Radiochemical Centre (Amersham, England); L-[U-^{14}C]phenylalanine, sp. act. 108 Ci/mol, from the Institute for Research, Production and Utilization of Radioisotopes (Prague, Czechoslovakia); pancreatic ribonuclease (5 × crystallized), lysozyme from egg white (3 × crystallized), 2-mercaptoethanol, spermidine trihydrochloride, 2,4-dinitrofluorobenzene (DNFB) and sodium dodecyl sulphate (SDS) from Sigma Chem. Corp. (St. Louis, Mo., U.S.A.); deoxyribonuclease (DNase), free from RNase (Worthington, Freehold, N.J., U.S.A.); Brij 58 from Atlas Chem. Co. (Wilmington, Del., U.S.A.); sodium *p*-chloromercuribenzoate (PCMB), soluble ribonucleic acid from *E. coli* B (tRNA), polyuridylic acid (poly U), potassium salt (lot 857 009) from Calbiochem. (Los Angeles, Calif., U.S.A.); 2,5-diphenyloxazole (PPO) and 1,4-bis(5-phenyloxazol 2-yl)-benzene (POPOP) from Packard Instrument Co. Inc. (Downers Grove, Ill., U.S.A.); millipore filters (0.45 μ) from Millipore Filter Corp. (Bedford, Mass., U.S.A.); Dowex 1 X10, Dowex 50 X4 (Fluka A.G., Buchs, Switzerland); formalin, reagent grade, from Zakłady Azotowe (Tarnów, Poland); D-(threo)chloramphenicol from Polfa (Kraków, Poland); [^{14}C]poly U was synthesized in this Institute by Mr. L. Nowak by the method of Basilio & Ochoa (1963), (Nowak & Szer, 1967) and [^{14}C]RNA from phage f2 was prepared by Mr. W. Filipowicz (to be published). Other chemicals were reagent grade products of Polskie Odczynniki Chemiczne (Gliwice, Poland); sucrose was purified by shaking a 60% aqueous solution (w/v) with active charcoal and then with Dowex 1 X10 and Dowex 50 X4 to deprive it of absorption at 260 nm.

Buffers. Standard buffer: 0.01 M-tris-HCl - 0.014 M-magnesium acetate - 0.06 M-KCl - 0.006 M-2-mercaptoethanol, pH 7.8 (Nirenberg & Matthaei, 1961). Buffer TMN-1 M: 0.01 M-tris-HCl - 0.01 M-magnesium acetate - 1 M-NH$_4$Cl, pH 7.2.

Preparation of the sucrose density gradient. A 50-ml linear sucrose gradient was prepared as described by Martin & Ames (1961) using 10% and 40% sucrose solutions in the standard buffer.

Ribosomes were prepared from *E. coli* B as described by Perzyński & Szafrański (1967), additionally purified by washing three times with the standard buffer solution, then suspended in the same buffer (20 mg/ml) and stored at -40°C. For experiments, the samples of ribosomes were thawed and then used within two weeks; they were stored during this period at 0°C without appreciable loss of activity in binding the mRNA's studied.

Preparation of the [14C]*poly U-ribosome complex.* [14C]Poly U, 15 µg (about 29 000 counts/min) was incubated with 3 mg of ribosomes in 1 ml of the standard buffer at 23°C for 6 min. Then the sample was cooled to 0°C, applied on 0.2 M-sucrose in standard buffer and centrifuged at about 2°C for 3 h at 140 000 g in a MSE Superspeed 50 ultracentrifuge (rotor 3×5 ml). To the sedimented [14C]poly U-ribosome complex 0.5 ml of the standard buffer was added and the sample was left at 0°C for a few hours, until the sediment became dissolved. The experiments on the stability of the complex were carried out on the same day.

Preparation of [14C]*polyribosomes. E. coli* B was grown in 100 ml of the minimal medium *C* of Roberts, Cowie, Abelson, Bolton & Britten (1955). The cells were centrifuged at the exponential phase of growth, when E_{650} was 0.6; then the cells were suspended in 30 ml of the same medium and incubated with 100 µCi of [14C]adenine at 36°C for 30 sec. Further steps of the preparation were carried out according to Hotham-Iglewski & Franklin (1967), with some modifications. Incorporation of [14C]adenine was stopped by adding sodium azide and chloramphenicol, and pouring the culture on the frozen minimal medium containing the two above compounds. The cells were harvested by centrifugation, digested with lysozyme and disintegrated with Brij 58 detergent in the presence of DNase. The homogenate was centrifuged three times for 5 min at 10 000 g and from the obtained supernatant the polyribosomes were isolated by sucrose-gradient centrifugation (Fig. 1). About 50% of the ribosome-bound radioactivity sedimented faster than 70s, corresponding to polyribosomes; the fractions no. 5 - 25 were pooled and stored at -40°C in small portions.
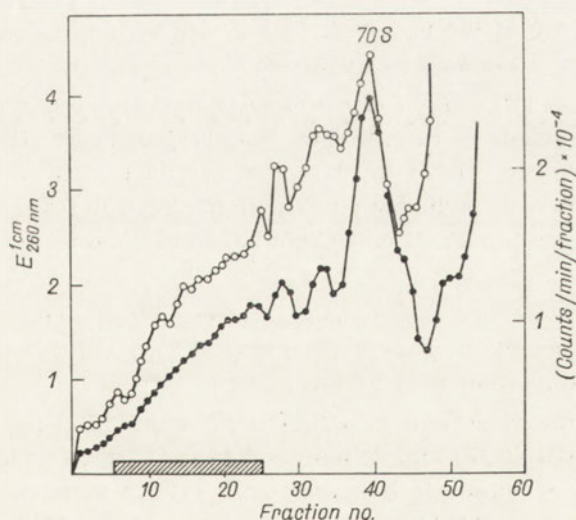

Fig. 1. Sedimentation pattern of *E. coli* cell-free extract in sucrose gradient. The extract, about 120 E_{260} units, was applied on 50 ml of the sucrose gradient (10 - 40%) and centrifuged at 2 - 5°C for 3 h in a Spinco L2 ultracentrifuge with SW-25.2 rotor. After centrifugation, fractions of about 0.9 ml were collected, beginning from the bottom of the tube. ●, Extinction at 260 nm determined at 1 cm light-path; ○, radioactivity assayed in samples of 20 µl taken from successive fractions.

The content of [^{14}C]mRNA in the polyribosome preparation was assayed by short digestion with RNase. It was found (Table 1) that RNase released 90% of the radioactivity of [^{14}C]polyribosomes. As ribosomal RNA is not susceptible to RNase under the conditions applied (Takanami, Yan & Jukes, 1965), it may be assumed that the released activity corresponds to mRNA.

Table 1

Digestion of [^{14}C]*polyribosomes with RNase*

The incubation mixture contained in 0.3 ml of the standard buffer 40 μl of polyribosome preparation and 1 μg of RNase. After 10 min at 25°C, the radioactivity of the mRNA-ribosome complex was determined as described in Methods.

Conditions	Radioactivity of the mRNA-ribosome complex	
	counts/min	%
Control (without incubation)	759	100
Incubation without RNase	648	85
Incubation with RNase	86	11

Preparation of E. coli [^{14}C]*mRNA from* [^{14}C]*polyribosomes.* The [^{14}C]poly-ribosome fraction was shaken with PVS (2.5 μg/ml), SDS (0.5%) and EDTA (0.03 M) for 2 min at 0°C. The mixture was deproteinized with an equal volume of 90% phenol at 4°C for 15 min. The water phase was collected, the phenol removed with ether, and the latter by bubbling nitrogen. The obtained preparation was purified on a Sephadex G-100 column at 4°C as described by Perzyński & Szafrański (1967). Radioactivity of the high-molecular [^{14}C]RNA amounted to 720 counts/min/μg. The preparation was stored at -40°C.

Determination of [^{14}C]*mRNA bound to ribosomes.* This was carried out by the millipore-filter technique as described by Nirenberg & Leder (1964) for studying the binding of aminoacyl-tRNA to ribosomes. For the experiments with [^{14}C]poly U-ribosome complex, the millipore filters were treated with 0.5 M-KOH for 15 min at 26°C to prevent adsorption of poly U not bound to ribosomes (Smolarsky & Tal, 1970a).

Treatment of ribosomes with formaldehyde and DNFB. The ribosomes were treated under the conditions given by Moore (1966b) except that the reaction with DNFB was carried out only for 2 h instead of 5 h.

Treatment of ribosomes with PCMB. The ribosomes, 200 μg, were incubated with 0.25 mM-PCMB at 37°C for 15 min, and to make sure of their reaction with PCMB, the ability of ribosomes to bind aminoacyl-tRNA in the presence of poly U was checked according to McAllister & Schweet (1968). Under the conditions applied, the binding was inhibited by 90% and it was completely restored on addition of an excess of 2-mercaptoethanol.

Analytical methods. Protein was determined by the method of Lowry, Rose-brough, Farr & Randall (1951). Concentration of ribosomes was determined by

measuring the extinction at 260 nm, taking $E_{1\,cm}^{1\%} = 150$ (Petermann, 1964). Radio-activity was determined in the Tri-Carb scintillation counter (Packard) with an efficiency of 60% for ^{14}C.

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

<div align="center"><i>Effect of cations on the stability of mRNA-ribosome complex</i></div>

In view of the possible role of magnesium bridges in formation of the mRNA-ribosome complex, the effect of EDTA-Na on the stability of [^{14}C]polyribosomes was studied. As shown in Table 2, in the presence of EDTA only about 50% of [^{14}C]polyribosome radioactivity was released. If magnesium alone were responsible for the stability of the mRNA-ribosome complex, a greater release of mRNA should be expected. However, not only 0.01 M-EDTA but also a five times higher concentration of the reagent and prolonged incubation up to 2 min, gave practically the same results. Thus it seems that magnesium is not directly involved in the attachment of mRNA to ribosomes. To check this supposition, the effect of magnesium, potassium, lithium and spermidine on the stability of polyribosomes was studied.

<div align="center">Table 2</div>

<div align="center"><i>Effect of EDTA on the stability of [^{14}C]polyribosomes</i></div>

Polyribosomes, 20 µl, were incubated for 1 min at 0°C in 0.3 ml of the standard buffer in which magnesium and potassium salts had been replaced by the indicated concentration of EDTA-Na (pH 7). The amount of [^{14}C]mRNA bound to ribosomes was determined as described in Methods.

Conditions	Radioactivity of mRNA bound to ribosomes	
	counts/min	%
Standard buffer (control)	378	100
0.01 M-EDTA	194	51
0.05 M-EDTA	221	58

From the results presented in Table 3 it may be seen that the absence of potassium in the incubation mixture which contained an appropriate concentration of magnesium salts, did not result in dissociation of the mRNA-ribosome complex as compared with the control sample. On the other hand, in the presence of 0.06 M and 0.12 M-K^+ ion the absence of magnesium resulted in only partial (30%) dissociation of the complex. This indicates that potassium ion is to a large extent able to replace magnesium ion in maintaining the mRNA-ribosome complex. Lithium had a similar effect. The absence of both magnesium and potassium in the incubation medium resulted in complete dissociation of polyribosomes indicating that cations are indispensable for the stability of the mRNA-ribosome complex. It should be noted that spermidine was able to replace completely Mg^{2+}, which confirms the lack of specificity of magnesium in the interaction of mRNA with ribosomes.

The effect of Mg^{2+} and K^+ on the stability of polyribosomes over the pH range 5 - 9.5, was also studied. From the stability curves for the mRNA-ribosome complex

Table 3

The effect of magnesium, potassium, lithium and spermidine on the stability of [¹⁴C]polyribosomes

Polyribosomes, 20 µl in standard buffer, were diluted with 3 ml of tris-HCl buffer, pH 7.8, containing chloride salts of the indicated cations, and incubated at 0°C for 1 min. The amount of [¹⁴C]mRNA bound to ribosomes was determined as described in Methods.

Addition	Radioactivity of mRNA bound to ribosomes	
	counts/min	%
Mg^{2+}, 0.014 M and K^+, 0.06 M (control)	378	100
Mg^{2+}, 0.14 M	361	95
K^+, 0.06 M	263	69
K^+, 0.12 M	270	71
Li^+, 0.06 M	260	69
Spermidine, 0.01 M	370	98
None	4	1

shown in Fig. 2 it appears that magnesium ion stabilized the complex over the pH range studied. On the other hand, in the presence of potassium ion only, polyribosomes gradually dissociated with increasing pH value. At pH 5 potassium replaced completely magnesium ion, but at pH 7 about 30% and at pH 9.5 50% of polyribosomes underwent dissociation. These results indicate that although magnesium is not directly involved in formation of mRNA-ribosome complex, it plays a decisive role in its stability.
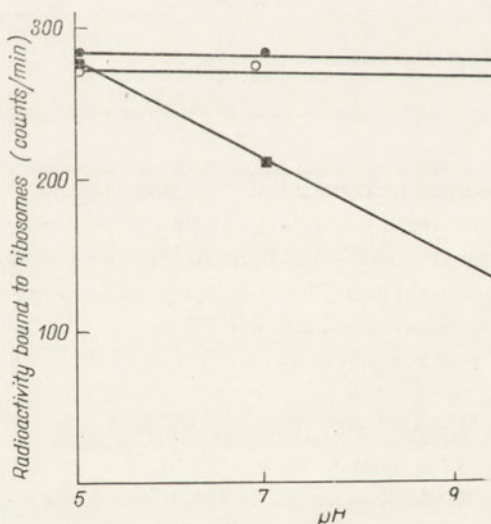

Fig. 2. Effect of pH on the stability of [¹⁴C]polyribosomes in the presence of Mg^{2+} and K^+. The incubation mixture consisted of 15 µl of polyribosome preparation (280 counts/min) in 3 ml of 0.05 M-tris-acetate buffer, containing: ●, 0.014 M-Mg^{2+} - 0.06 M-KCl; ○, 0.014 M-Mg^{2+}; ■, 0.06 M-K^+. The cations were added as acetate salts. The incubation was for 1 min at 0°C.

This conclusion was confirmed in the experiments with urea (Table 4) which is known to destroy macromolecular conformation. The polyribosomes dissociated more readily under the influence of 1 M-urea in the presence of K^+ alone than in the presence of Mg^{2+}. The complex appeared to be most stable in the presence of both these ions together, and it underwent dissociation when the concentration of urea was raised to 4 M.

Table 4

Effect of urea on the stability of [^{14}C]*polyribosomes in the presence of* Mg^{2+} *and* K^+

The incubation mixture contained 15 µl of the polyribosome preparation and the indicated concentrations of urea in 3 ml of the standard buffer or buffer deprived of Mg^{2+} or K^+. Incubation 1 min at 0°C.

Urea	Cation	Radioactivity of mRNA bound to ribosomes	
		counts/min	%
None	Mg^{2+}, K^+ (control)	286	100
1 M	Mg^{2+}, K^+	256	90
4 M	Mg^{2+}, K^+	57	20
1 M	Mg^{2+}	173	64
1 M	K^+	84	39

A similar series of experiments has been carried out on the complex of a synthetic mRNA with ribosomes. The results concerning the effect of urea and Mg^{2+} and K^+ on the stability of the [^{14}C]poly U-ribosome complex, are summarized in Table 5. The complex was resistant to 1 M-urea in the presence of Mg^{2+} and K^+

Table 5

Effect of urea and Mg^{2+} *and* K^+ *ions on the stability of the* [^{14}C]*poly U-ribosome complex*

The poly U-ribosome complex, 20 µl, was incubated for 1 min at 0°C in 3 ml of the standard buffer, or buffer containing no Mg^{2+} or K^+, and the indicated concentration of urea. The amount of [^{14}C]poly U bound to ribosomes was determined as described in Methods.

Urea	Cation	Radioactivity of poly U bound to ribosomes	
		counts/min	%
None	Mg^{2+}, K^+ (control)	238	100
1 M	Mg^{2+}, K^+	220	92
4 M	Mg^{2+}, K^+	34	14
1 M	Mg^{2+}	240	100
None	Mg^{2+}	230	97
None	K^+	50	21

together and in the presence of Mg^{2+} alone, indicating that potassium is not essential for the stability of the complex. The absence of Mg^{2+}, even without the addition of urea, resulted in an almost complete dissociation of the complex. Thus in the case of the poly U-ribosome complex, in contrast to the polyribosomes (Table 3), monovalent cation was unable to replace magnesium.

Role of ribosome structure in binding of mRNA

The effect of washing of ribosomes with 1 M-NH_4Cl and blocking of their amino and thiol groups, on the interaction with *E. coli* mRNA, f2 RNA and poly U, was studied. In the preliminary experiments, the optimum conditions were determined for the binding of the templates with untreated ribosomes (ribosomes washed three times with standard buffer, see Methods). The optimum temperature for the interaction of ribosomes with *E. coli* [^{14}C]mRNA was found to be about 23°C (Fig. 3). From the data of other authors it appears that binding of poly U to ribo-
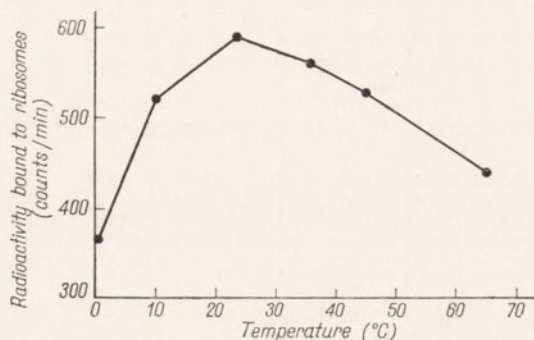
Fig. 3. Effect of temperature on the binding of *E. coli* [^{14}C]mRNA to ribosomes. The incubation mixture contained in 0.25 ml of the standard buffer 400 µg of ribosomes and [^{14}C]mRNA (5000 counts//min). The samples were incubated for 6 min at the indicated temperature.

somes is not dependent on temperature when this exceeds 8°C (Barondes & Nirenberg, 1962; Takanami & Okamoto, 1963; Logan & Whitmore, 1966; Moore, 1966a). Similarly, binding of f2 RNA to ribosomes is of the same order over the temperature range 10 - 37°C (Takanami *et al.*, 1965). On the basis of these results, all further experiments on mRNA binding to ribosomes were carried out at 23°C.

Binding of *E. coli* mRNA, f2 RNA and poly U as a function of ribosome concentration, is shown in Fig. 4 and 5. The saturation curve of *E. coli* [^{14}C]mRNA
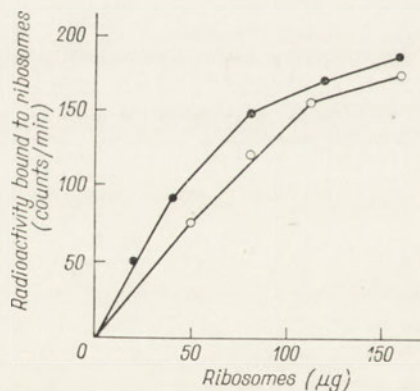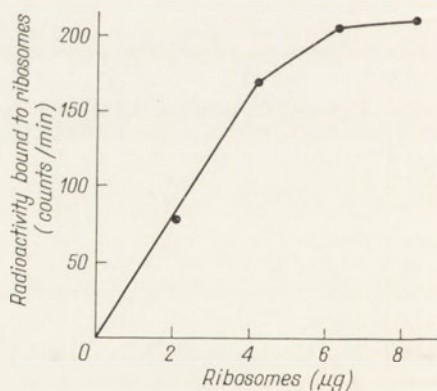

Fig. 4   Fig. 5

Fig. 4. Binding of ○, *E. coli* [^{14}C]mRNA and ●, f2 [^{14}C]RNA to ribosomes. *E. coli* mRNA (440 counts/min) or f2 RNA (1.27 µg corresponding to 222 counts/min) was incubated in the standard buffer (final volume 0.25 ml) with different amounts of ribosomes for 6 min at 23°C.

Fig. 5. Binding of [^{14}C]poly U to ribosomes. Poly U (0.48 µg corresponding to 550 counts/min) was incubated with different amounts of ribosomes. Conditions of incubation as in Fig. 4.

with ribosomes reached the maximum when the amount of ribosomes was 112 µg, and at this point 30% of the mRNA applied was bound to ribosomes (Fig. 4). In the case of f2 [^{14}C]RNA binding, about 70%, i.e. about 0.8 pmole of RNA (mol. wt. of f2 RNA 1.1×10^6 according to Strauss & Sinsheimer, 1963) was bound to 30 pmoles of ribosomes (mol. wt. of ribosomes 2.6×10^6 according to Tissieres, Watson, Schlessinger & Hollingworth, 1959). Assuming, according to Takanami *et al.* (1965) that only monosomes were formed in this reaction, it should be accepted that only about 3% of the ribosomes used in the experiments were able to bind f2 RNA. The binding of [^{14}C]poly U to ribosomes was the greatest when their weight ratio was about 0.09:1; under these conditions 33% of the applied poly U became bound to ribosomes. From the calculations of Moore (1966a) it appears that, irrespective of the molecular weight of the poly U, at least 80% of ribosomes are able to interact with this template. This indicates that poly U possesses a much higher affinity to ribosomes than natural mRNA.

The effect of ribosome structure on the interaction with different templates was studied at the optimum template to ribosomes ratio. Washing of ribosomes with 1 M-NH$_4$Cl, as demonstrated by Iwasaki, Sabol, Wahba & Ochoa (1968), removes initiation factors and other proteins. In our experiments, the ribosomes which were previously washed three times with standard buffer, were suspended in TMN-1 M buffer, shaken gently for 12 h at 4°C, and centrifuged at 140 000 g for 2.5 h. The upper two-thirds of the supernatant was collected, and the amount of protein washed out from ribosomes was determined. The pellet of sedimented ribosomes was suspended in standard buffer and used for binding of poly U and natural mRNA's. Washing of ribosomes with 1 M-NH$_4$Cl resulted in removal of 8% of their protein (Table 6). These proteins appeared to be essential both for

Table 6

Effect of washing of ribosomes with 1 M-NH$_4$Cl *on binding of natural templates and poly U*

The incubation mixture contained in 0.3 ml of the standard buffer: 1) 120 µg of ribosomes and *E. coli* [^{14}C]mRNA (440 counts/min); or 2) 90 µg of ribosomes and phage f2 [^{14}C]RNA (220 counts /min); or 3) 5 µg of ribosomes and [^{14}C]poly U (550 counts/min). Incubation for 6 min at 23°C.

Ribosomes	Percentage of protein washed out from ribosomes	Radioactivity bound to ribosomes (counts/min)		
		E. coli mRNA	f2 RNA	poly U
Non-washed (control)	1	160	160	180
Washed with 1 M-NH$_4$Cl	8	40	20	250

the binding of *E. coli* mRNA and phage f2 RNA. On the other hand, the binding of poly U to ribosomes increased after their washing with 1 M-NH$_4$Cl.

The results of experiments on the possible involvement of amino groups and thiol groups in binding of mRNA to ribosomes, are summarized in Table 7. The ribosomes treated with formaldehyde, which reacts with amino and thiol groups of

Table 7

Effect of treatment of ribosomes with HCOH, DNFB and PCMB on the binding of poly U and natural templates

The ribosomes were treated with the indicated reagents as described in Methods. Conditions of binding of mRNA to ribosomes as in Table 6.

Ribosomes treated with	Radioactivity bound to ribosomes (counts/min)		
	E. coli mRNA	f2 RNA	poly U
None (control)	165	145	180
Formaldehyde	0	0	0
DNFB	30	10	160
PCMB	146	120	215

proteins and with the amino groups of nucleic acids (Fraenkel-Conrat, 1954, 1957) lost completely the ability to react with all the templates studied. The ribosomes treated with DNFB, which under the conditions applied reacts specifically with amino and thiol groups of proteins (Moore, 1966b) were unable to bind natural mRNA's but retained the ability to bind poly U. The treatment of ribosomes with PCMB proved that thiol groups are not involved in the binding of either type of template. From these results it may be concluded that amino groups of ribosomal RNA are essential for the attachment of poly U to ribosomes, whereas binding of natural mRNA's requires the participation at least of free amino groups of ribosomal protein(s).

To elucidate whether the binding site on the ribosome is common for the natural and synthetic templates, binding of phage f2 [^{14}C]RNA to ribosomes was studied
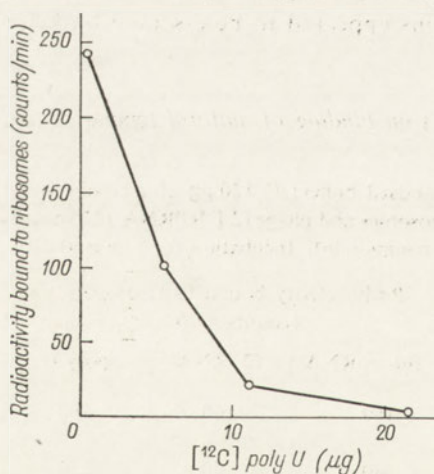

Fig. 6. Competition between f2 RNA and poly U n the interaction with ribosomes. The incubation mixture contained in 0.25 ml of the standard buffer 1.8 μg of f2 [^{14}C]RNA (315 counts/min), 160 μg of ribosomes and the indicated amounts of [^{12}C]poly U. Incubation for 6 min at 23°C.

in the presence of poly U (Fig. 6). The binding of radioactivity was completely inhibited by an excess of poly U, which indicates that poly U and phage f2 RNA were bound to the same site on the ribosomes. This conclusion was confirmed by the results of the experiments on the effect of poly U on translation of f2 RNA in the *E. coli* Q13 cell-free system. On addition of poly U the translation was inhibited by 90 %.

DISCUSSION

The attachment of mRNA to ribosomes, an initial step in the translation process, has been studied in a number of laboratories. However, the results obtained have not fully elucidated the mechanism of this interaction. Binding of mRNA to ribosomes by complementary base pairing with rRNA does not seem to be involved since poly-5,6-dihydro U, unable to form hydrogen bonds, is nevertheless bound to ribosomes (Szer & Nowak, 1967). Thus it is likely that the phosphate residues rather than mRNA bases participate in the binding. In this case, binding would require the participation of divalent cations, especially magnesium, which is present in bacterial cells at a considerable, about 30 mM, concentration (Lubin & Ennis, 1964). The role of magnesium would consist both in neutralization of the negatively charged phosphate groups and in formation of the ionic linkage. The results of our experiments indicate that in the binding of natural mRNA's to ribosomes magnesium can largely be replaced by monovalent cations, whereas in case of poly U divalent cations seem to be essential although they can be replaced by spermidine (Moore 1966a). The linkages between natural mRNA and ribosomes formed in the presence of different cations are not equivalent. As demonstrated by our experiments, replacement of magnesium by monovalent cations decreases the stability of polyribosomes. Thus it seems that the role of magnesium does not consist in formation of ionic bridges but rather in maintaining an appropriate structure of the ribosomes required for stability of the complex.

The other type of binding of mRNA to ribosomes may be of the hydrogen bond type. It could result from the reaction between the phosphate groups of mRNA and the amino groups of rRNA or amino or thiol groups of ribosomal proteins. The role of ribosomal protein in the binding of mRNA's has been studied in a number of laboratories. It has been demonstrated that binding of natural mRNA to ribosomes requires the participation of initiation protein factors which may be eluted from ribosomes at high salt concentration. On the other hand, these factors are not essential for the binding of synthetic mRNA, e.g. poly U (Iwasaki *et al.*, 1968; Brown & Doty, 1968; Maitra & Dubnoff, 1968; Herzberg, Lelong & Revel, 1969). However, the studies of Zak, Nair & Rabinowitz (1966) and Kaji, Suzuka & Kaji (1966) on tryptic digestion of ribosomes, as well as experiments of Traub & Nomura (1968) and Smolarsky & Tal (1970b) on the structure and function of ribosomes, indicate the involvement of some proteins other than the initiation factors in poly U interaction with ribosomes. On the contrary, the experiments of Moore (1966b) on the active groups of ribosomes suggest that neither amino nor thiol groups of ribosomal proteins participate in this process. It may, therefore, be assumed that proteins are indirectly involved in the interaction of poly U with ribosomes by maintaining an appropriate conformation of rRNA. On the basis of Moore's experiments with poly U (Moore, 1966b) it has generally been assumed that mRNA's react with ribosomes through hydrogen bonds between the phosphate groups of mRNA and the amino groups of ribosomal RNA. Our experiments show that this is valid only in relation to poly U, as the binding of *E. coli* mRNA as well as of

phage f2 RNA requires the participation of amino groups of ribosomal proteins. However, we have also demonstrated a competition between f2 RNA and poly U in their interaction with ribosomes which indicates that both are bound to the same site on the ribosome. Thus, it seems unlikely that either type of template reacts with different groups on the ribosome. The differences observed in the mechanism of binding could be explained by different structures of both types of mRNA's. It is possible therefore that poly U, which possesses higher affinity to ribosomes than f2 RNA, can bind to them even after blocking of amino groups of the ribosomal proteins.

To conclude, it may be postulated that the physiological complex of mRNA with ribosomes is the result of hydrogen bond formation between the phosphate groups of mRNA and the amino groups of rRNA and ribosomal proteins.

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REAKCJE RYBOSOMÓW *ESCHERICHIA COLI* Z MATRYCAMI

Streszczenie

1. Kationy jednowartościowe w znacznym stopniu zastępują magnez w reakcji *E. coli* mRNA z rybosomami, podczas gdy w przypadku kompleksu poli U-rybosomy nie odgrywają tej roli.

2. Kompleks *E. coli* mRNA-rybosomy w obecności Mg^{2+} i K^+ równocześnie rozpada się dopiero pod wpływem 4 M-mocznika, natomiast w obecności tylko jednego z tych kationów jest wrażliwy już na 1 M-mocznik. Z drugiej strony 1 M-mocznik w obecności samego Mg^{2+} nie powoduje rozpadu kompleksu poli U-rybosomy.

3. Kompleks *E. coli* mRNA-rybosomy w obecności Mg^{2+} jest niewrażliwy na zmiany pH od 5 do 9,5.

4. Formylacja rybosomów znosi ich zdolność do reakcji zarówno z poli U jak i naturalnymi matrycami.

5. Zablokowanie grup aminowych białek rybosomowych uszkadza reakcję rybosomów z naturalnymi matrycami, nie wpływając w istotny sposób na ich reakcję z poli U.

A. RABCZENKO and D. SHUGAR

STUDIES ON THE CONFORMATION OF NUCLEOSIDES, DINUCLEOSIDE MONOPHOSPHATES AND HOMOPOLYNUCLEOTIDES CONTAINING URACIL OR THYMINE BASE RESIDUES, AND RIBOSE, DEOXYRIBOSE OR 2'-O-METHYLRIBOSE

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Circular dichroic spectra, in the range 220 - 330 nm, have been recorded for a series of monomers, dinucleoside monophosphates, and single-stranded and helical forms, of homopolynucleotides of uracil (and in several instances thymine), in which the carbohydrate moiety consisted of ribose, deoxyribose or 2'-O-methylribose. The temperature-dependence of the intensity of the long-wavelength positive CD band was examined for the entire series. Model building with Corey-Pauling-Koltun (CPK) models was employed to examine the validity of some of the findings.

For dinucleoside monophosphates of uracil, the B_{2u} band intensity, as well as the slope of the curve for its temperature-dependence, both increase in the order d(UpU), r(UpU), r(UmpU). Published data on photodimerization rates for the first two of these are in agreement with an increased degree of base stacking in the same order.

For the single-stranded polynucleotide pairs poly dU, poly rU and poly dT, poly rT, the increased intensity of the B_{2u} band for the ribo polymers relative to the deoxyribo holds only below a certain temperature. The CD data, regarded as a measure of base stacking, are again supported by literature data on photodimerization rates as a function of temperature. Poly Um, however, exhibits an appreciably higher B_{2u} band intensity than the foregoing at all temperatures, indicative of an even higher degree of base stacking.

The striking similarity between the entire CD spectra for the helical forms of poly rU, poly rT and poly Um, which exhibit differences only in band intensities, indicates that all three possess similar structures consisting of two strands with identical polarities. This proposal is supported by an examination of CPK models.

The role of the 2'-hydroxyl in ribopolynucleotides is discussed. A comparison with literature data for other polymers shows that the effect of a 2'-OH (or 2'-H or 2'-OMe) is dependent in part on the nature of the nitrogenous base. One of the resulting conclusions is the possibility, in poly rU, of hydrogen bonding between the 2'-OH of one residue to the $O_{4'}$ of the neighbouring ribose ring. Such a model would readily account for the reported high rigidity of single-stranded poly rU.

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Synthetic oligo- and polynucleotides have been extensively employed for studies on the degree and nature of the stacking interactions between the aromatic bases, optical activity (ORD and/or CD) being the method of choice, largely because of its relatively high sensitivity to interactions between the base chromophores, particularly in the case of oligonucleotides, where ordinary absorption methods give uncertain or negative results. Although the data obtained by these techniques are largely empirical, some attempts have been made to formulate theoretical interpretations for the optical activity of homo-oligonucleotides, e.g. oligo A[1] (Brahms, Michelson & Van Holde, 1966), and at least one procedure has been developed for the derivation of the ORD spectra of hetero-oligonucleotides from those obtained experimentally on dinucleoside monophosphates (Cantor, Jaskunas & Tinoco, 1966).

The present study is devoted to a detailed examination of the optical activity of dinucleoside monophosphates and polynucleotides of the ribose and deoxyribose forms of U and T, made possible in part by the recent preparation of poly dU (Żmudzka, Bollum & Shugar, 1969). Furthermore, since UV spectral data show that poly dT exhibits very little secondary structure, and poly dU none at all under conditions where both poly rU and poly rT form helical structures, this implicates in some way participation of the 2′-hydroxyl. Therefore advantage has been taken of the newly synthesized poly 2′-O-MeU (Żmudzka & Shugar, 1970), which is included in the present investigation.

MATERIALS AND METHODS

Uridine, ribosylthymine, and thymidine were Calbiochem (Los Angeles, Calif., U.S.A.) grade A products, checked prior to use by spectral and chromatographic procedures; 2′-O-methyluridine was obtained by deamination of 2′-O-methylcytidine (a gift from Dr. C. B. Reese) as described by Winkley & Robins (1968).

r(UpU) was a Waldhof (Mannheim, West Germany) product. A sample of r(UmpU) was kindly provided by Dr. B. G. Lane. d(TpT) and d(UpU) were prepared according to standard procedures as described by Gilham & Khorana (1958).

Poly rU was a Calbiochem product; poly dT was kindly provided by Dr. F. J. Bollum. The preparation of poly rT has been elsewhere described (Szer, Świerkowski & Shugar, 1963), as has that of poly dU (Żmudzka et al., 1969) and poly 2′-O-MeU (Żmudzka & Shugar, 1970).

All solutions were made up in glass-distilled water, and buffer reagents were of analytical grade. Concentrations of both monomers and polymers were estimated spectrophotometrically with the aid of a Zeiss (Jena) VSU-2 spectrophotometer, using known extinction coefficients. All pH measurements were performed with a Radiometer PHM 22 meter, using a glass semi-micro electrode.

CD spectra were obtained on solutions exhibiting optical densities below 2.0. Spectra usually covered the range 220 - 320 nm. The instrument employed was

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[1] Abbreviations used in this text conform to the nomenclature adopted by the Combined Commission on Biochemical Nomenclature of IUPAC-IUB (*J. Mol. Biol.* **55**, 299, 1971).

a JASCO ORD/UV-5 spectropolarimeter with circular dichroism attachment. The 10-mm pathlength cuvettes were mounted in a specially constructed thermo-electrically controlled block, the temperature of which could be varied continuously over the range from -11°C to 90°C and measured by means of a thermistor inserted directly into the cuvette. Recording of a spectrum was always followed by measurement of the background over the same wavelength range; and measurements were usually repeated several times in order to improve the accuracy. The instrument was calibrated with the S-1032 standard supplied by JASCO.

RESULTS

The overall results are presented below according to the system used, amongst others, by Miles, Robins, Robins, Winkley & Eyring (1969a); in this system, the observed $\pi \rightarrow \pi^*$ transitions of the bases are classified into three groups referred to as B_{2u}, B_{1u} and E_{1u}, by analogy with the nomenclature for the corresponding transitions in benzene. While this system of nomenclature has no real theoretical justification, it nonetheless possesses the advantages of simplicity and convenience, particularly in preparing comparisons with the results of other laboratories.

Quantitative data are presented in Table 1 for the circular dichroism of the compounds embraced in this report, all in neutral aqueous medium at 20°C. The CD spectra for the relevant compounds are exhibited in Fig. 1. The dependence of the CD spectra on temperature for the dinucleoside monophosphates is shown

Table 1

Circular dichroism spectral data, in 0.01 M-cacodylate buffer pH 6.9, at 20°C for monomers, dinucleoside monophosphates and homopolynucleotides

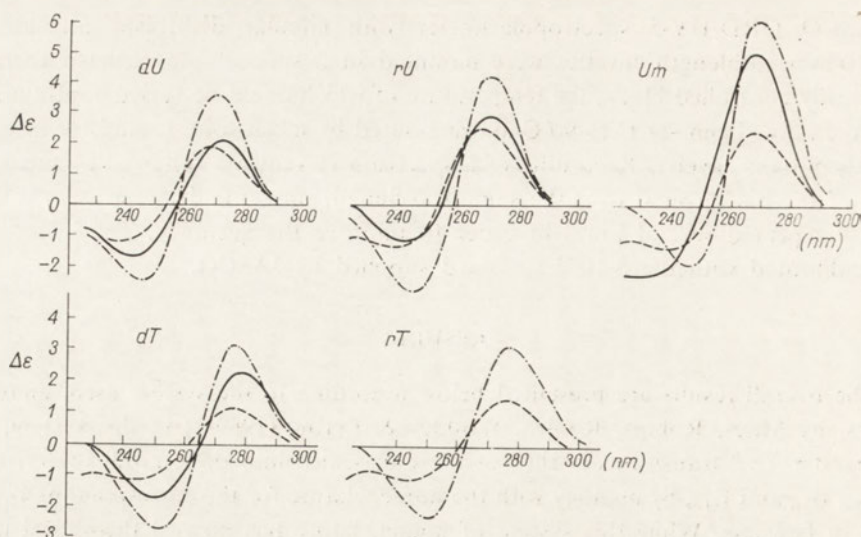| Band / Compound	B_{2u}		B_{1u}	
	λ_{max}	$\Delta\varepsilon_{max}$	λ_{max}	$\Delta\varepsilon_{max}$
poly dU	272	+3.6	248	—2.5
d(UpU)	272	+2.1	242	—1.8
dU	268	+1.8	237	—1.2
poly rU	272	+4.2	248	—2.9
r(UpU)	270	+2.9	243	—1.3
rU	268	+2.5	240	—1.4
poly Um	270	+6.1	246	—2.1
r(UmpU)	269	+5.0	235*	—2.3
Um	268	+2.5	240	—1.4
poly dT	276	+3.2	252	—2.9
d(TpT)	278	+2.3	251	—1.8
dT	272	+1.2	243	—1.1
poly rT	276	+3.4	252	—2.5
r(TpT)	—	—	—	—
rT	272	+1.5	243	—1.2

* Approximate value only.

Fig. 1. CD spectra of nucleosides, dinucleoside monophosphates, and single-stranded forms of polynucleotides, containing uracil or thymine residues, in 0.01 м-cacodylate buffer, pH 6.9, 20°C: — — —, monomers; ———, dinucleoside monophosphates; —·—·—, single-stranded poly-nucleotides.

in Fig. 2, and the analogous data for the polynucleotides in Fig. 3. The CD values for temperatures below 0°C were obtained on solutions containing 3 м-KF. Both our own observations, and those previously reported by Brahms, Maurizot & Michelson (1967), indicate no observable differences in the CD spectra of dinucleoside monophosphates in aqueous 3 м-KF as compared to those in 0.01 м-sodium cacodylate used in the present study.
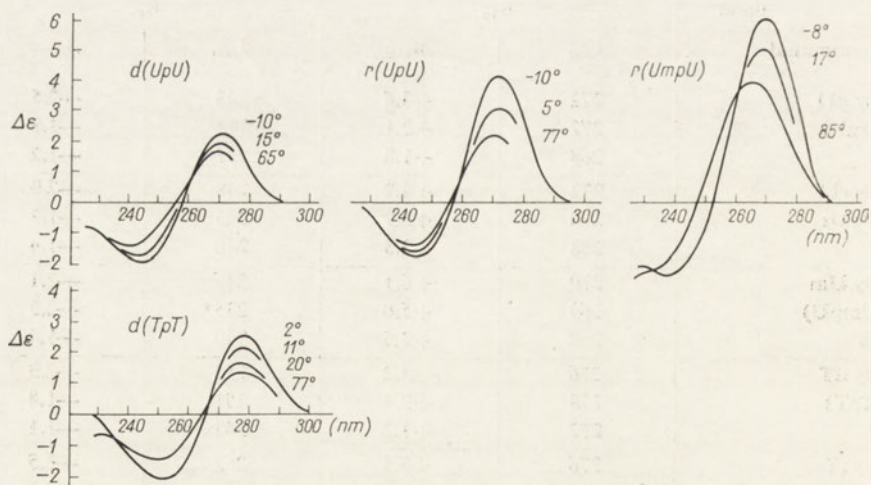
Fig. 2. CD spectra for dinucleoside monophosphates at various temperatures (°C), in 0.01 м-cacody-late buffer, pH 6.9. Measurements below 0°C were conducted on solutions containing 3 м-KF (see text for further details).

The shapes of the CD spectra, and the locations of the individual bands, are in reasonable agreement with those reported by Brahms *et al.* (1967), Hashizume & Imahori (1967), Wolfe, Oikawa & Kay (1969) and Miles *et al.* (1969a). However, the intensities of specific bands herein reported are somewhat lower than those recorded by Brahms *et al.* (1967), but in reasonably good agreement with the measurements of Miles *et al.* (1969a).

An examination of the foregoing figures shows that the various compounds, all of which contain the 2,4-diketopyrimidine chromophore, possess in common two well-defined bands: one positive (in the range 260 - 290 nm) referred to as B_{2u}, the other negative (in the range 230 - 260 nm) referred to as B_{1u}. In addition, each exhibits in the wavelength range 205 - 230 nm the "tail" of a band denoted by Miles *et al.* (1969a) as E_{1ua}. Furthermore, in passing from the monomer to the
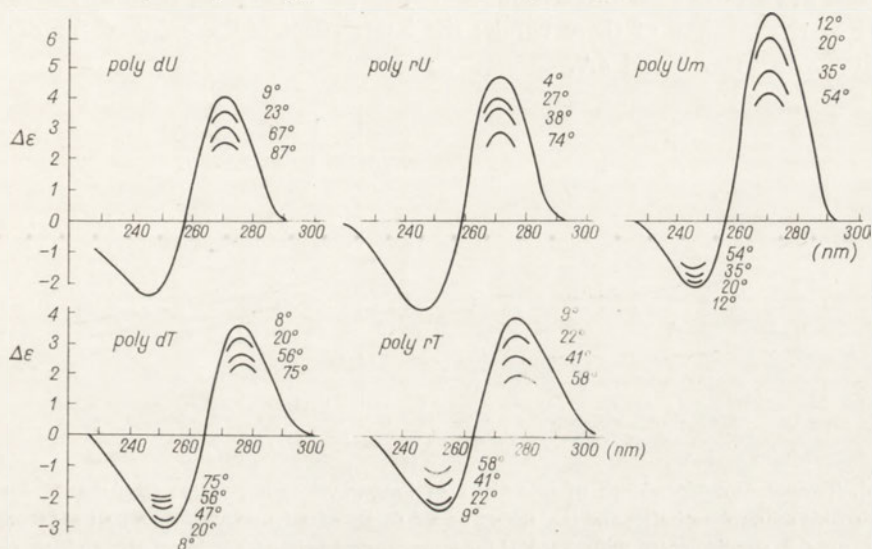

Fig. 3. CD spectra for single-stranded forms of polynucleotides at various temperatures (°C), in 0.01 M-cacodylate buffer, pH 6.9.

dinucleoside monophosphate and the single-stranded forms of the polymers, the increases in intensity of the individual bands, except for d(UpU), are accompanied by corresponding wavelength shifts to the violet in the maxima of these bands.

A comparison of the spectra for the various compounds examined leads also to the following generalizations:

(a) In the case of all four monomers (Fig. 1). the "tail" of the negative E_{1ua} band is partially overlapped by the negative B_{1u} band.

(b) For the dinucleoside monophosphates, with the exception of r(UmpU), the negative E_{1ua} band is shifted to the violet so that it is readily differentiated from the negative B_{1u} band (Fig. 1).

(c) Whereas the intensity of the B_{2u} band varies for the different dinucleoside monophosphates, increasing in the order d(TpT), d(UpU), r(UpU), r(UmpU), that for the B_{1u} band is affected to only a small degree.

(d) The intensities of the individual bands for the single-stranded "random coil" forms of the polymers are essentially similar to those for the corresponding dinucleoside monophosphates, but exhibit an increased intensity.

Temperature dependence of Cotton effect. The CD spectra of all the mononucleoside diphosphates and polynucleotides exhibit temperature dependence. If any such effect exists in the case of the monomer units, it is below the level of instrumental errors; reference is made to this because of the previous observation (Wróbel, Rabczenko & Shugar, 1970) on the small, but definite, variations in the absorption spectra of the neutral and cationic forms of cytidine and cytidine-5′-monophosphate as a function of temperature, most likely due to modifications in solvatation energy.

In general, with increase in temperature, there is a decrease in intensity of the B_{2u} and B_{1u} bands of the dinucleoside monophosphates, accompanied by wavelength shifts of the maxima of these bands; the latter effect is less marked for d(TpT), as shown in Figs. 2 and 4A.
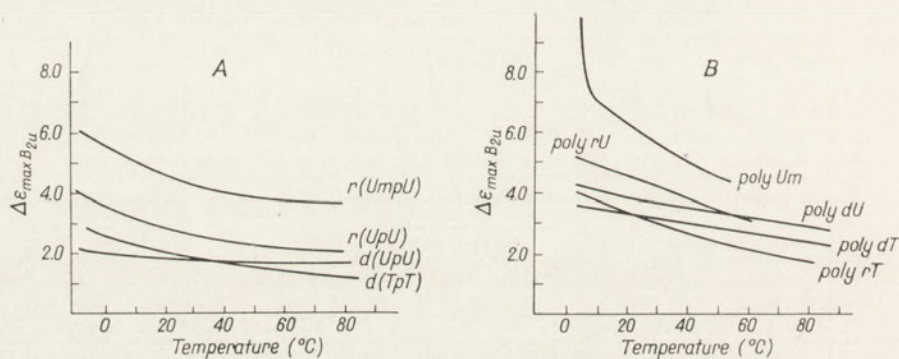

Fig. 4. Temperature-dependence of intensities of long-wavelength positive (B_{2u}) bands for: *A*, dinucleoside monophosphates, and *B*, single-stranded forms of polynucleotides, in 0.01 M-cacodylate buffer, pH 6.9, supplemented with 3 M-KF for temperatures below 0°C. *Note*: the sudden rise in B_{2u} intensity for poly Um below 10°C is due to formation of the helical structure.

It should be noted that, for each of the dinucleoside monophosphates, the CD curves at the different temperatures exhibit an "isosbestic" point in the neighbourhood of 260 - 265 nm. Similar "isosbestic" points were recorded by Brahms *et al.* (1966) for ApA and higher oligonucleotides of rA, but apparently no significance was attached to this, since the author does not comment upon it. Notwithstanding the 5% error inherent in the recording of the spectra, we are inclined to believe that these isosbestic points are real. Attention is drawn to the fact that whereas, with increasing temperature, the spectra of the dinucleoside monophosphates approach those of the respective monomers, coincidence of the two is not reached even at elevated temperatures.

For the corresponding polymers (Fig. 3), elevation of the temperature only slightly affects the location of the maxima of the CD bands; the principal consequence is a decrease in intensity of the bands.

CD spectra of structured polynucleotides. In those instances where a given polymer is capable of forming a helical structure (poly rU, poly rT, poly Um), the CD spectrum is similar in all instances (Fig. 5), irrespective of whether the agent employed
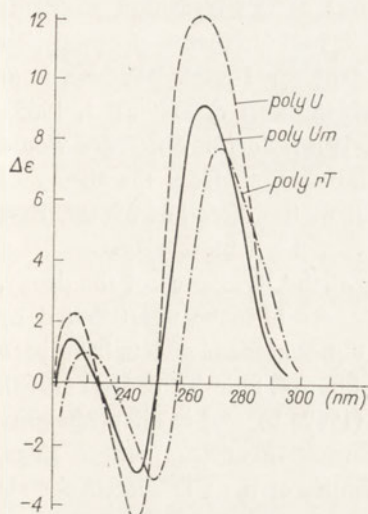

Fig. 5. CD spectra in 0.01 M-cacodylate buffer, pH 6.9, at 9°C, for the helical forms of: — — —, poly rU with 10^{-4} N-spermidine; ————, poly Um with 0.2 M-Na$^+$; —·—·—, poly rT with 0.01 M-Mg^{2+}.

for formation of the helical form is monovalent Na$^+$, the divalent cation Mg^{2+}, or a polyamine. The intensities of the various bands are also of the same order of magnitude.

Influence of a 5-methyl substituent. With the exception of the monomers (which will be discussed below), introduction of a 5-methyl substituent results only in an approximately 5 nm shift of the band maxima, with no appreciable modification of band intensities. This is in agreement with the known bathochromic shift in the UV spectrum of a uracil residue on introduction of a 5-methyl substituent to give thymine (Shugar & Fox, 1952).

DISCUSSION

For the sake of simplicity, we shall take up in turn the properties of monomers, dinucleotides, single-stranded polynucleotides, the ordered structural forms of the latter, and, finally, the role of the 2'-hydroxyl in polynucleotide conformation.

Monomers. A comparison of the CD spectra of the monomers reveals two points of interest. First, the pronounced difference between dT (and rT) and dU (and rU). The differences in location of the various bands between the two are, of course, due to the inductive effect of the 5-methyl substituent, as reflected in the bathochromic shift of the UV spectrum of thymine nucleosides relative to uracil nucleosides. More striking is the twofold lower intensity of the B_{2u} band of thymine nucleosides as compared to the corresponding uracil nucleosides (Fig. 1). According to the rules formulated by Miles, Robins, Robins, Winkley & Eyring (1969b), this should be ascribed to differences in the angles χ_{CN} for the two nucleosides; but theoretical considerations (Lakshminarayanan & Sasisekharan, 1969a) argue against

this; furthermore there is no reason why there should be differences in conformation between the pentose rings in dT and dU (or rT and rU), in agreement with the near identity of $J(H_{1'}-H_{2'})$ for dT (Lemieux, 1961) and dU (Prestegard & Chan, 1969). More important are the differences in the directions of the transition moments of the B_{2u} band in thymine relative to that for uracil. Measurements of absorption on oriented crystals show that the transition moment for 1-methylthymine is at an angle of -19° with respect to the $N_{(1)}$—$C_{(4)}$ axis (Stewart & Davidson, 1963), whereas for 1-methyluracil it is 0° (Eaton & Lewis, 1970). Such a difference would account for the observed modifications in rotational strength from the theory of Tinoco (1964), which relates the rotational strength to the direction of the base transition moment with respect to the polarizability of the pentose ring.

The second point of interest is the low intensity of the B_{2u} bands of the deoxy, as compared to the ribo, nucleosides. Such differences have been attributed by Warshaw & Cantor (1970) to experimental errors, but analogous effects have been observed by Emerson, Swan.& Ulbricht (1967), Nishimura, Shimizu & Iwai (1968), Adler, Grossman & Fasman (1969) and Miles *et al.* (1969a,b), and in all probability these effects are due largely to differences in conformation of the pentose rings, resulting in modification of the angles χ_{CN}. The identity of the CD spectra for rU and Um (Fig. 1 and Table 1) therefore points to the identity of χ_{CN} for both these nucleosides. Bobst, Rottman & Cerutti (1969b) also noted identical CD spectra for 5'-AMP and 5'-AmMP; while the low intensity of the bands in this case raises some doubt as to the accuracy, this observation was substantiated by the demonstration that the coupling constants $J(H_{1'}-H_{2'})$ are practically identical.

One further point should be emphasized, viz. that the B_{1u} bands for *all* the nucleosides (Fig. 6) exhibit almost identical shapes and intensities. We are unable at the moment to comment on this regularity.
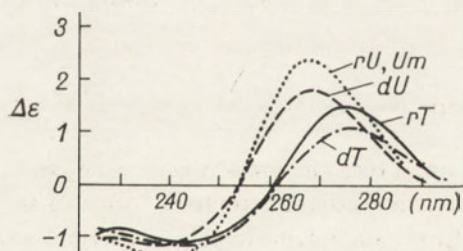

Fig. 6. CD spectra of rU and Um, dU, rT and dT in 0.01 M-cacodylate buffer, pH 6.9, at 20°C.

Dinucleoside monophosphates. The CD spectra for the series r(UpU), r(UmpU) and d(UpU), notwithstanding the identity of their UV absorption spectra, exhibit marked differences (Fig. 5). They likewise differ as regards the temperature dependence of the B_{2u} band intensities. The differences between r(UpU) and d(UpU) are reflected in their photochemical behaviour; irradiated r(UpU) forms cyclobutane photodimers in higher yield than d(UpU) (Brown, Freeman & Johns, 1966), testifying to more extensive base stacking in the former. This implies an even higher degree of base stacking in r(UmpU), the B_{2u} band of which is the most intense. The dependence of extent of photodimerization on degree of stacking is equally illustrated

by the decrease in photodimer yield in irradiated d(TpT) with increase in temperature (Tramer, Wierzchowski & Shugar, 1969), which parallels the decrease in B_{2u} band intensity of d(TpT) with increase in temperature (Fig. 4).

In the light of the foregoing, there appears to be a clear correlation between the CD spectra (Fig. 7) and degree of base stacking in dinucleoside monophosphates.
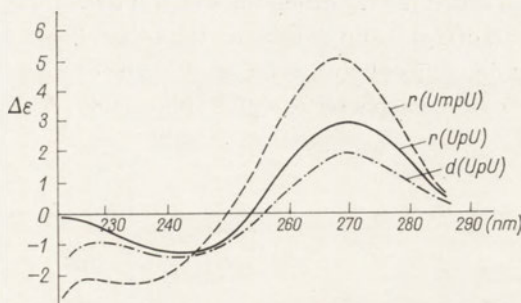

Fig. 7. CD spectra of r(UpU), r(UmpU) and d(UpU) in 0.01 M-cacodylate buffer, pH 6.9, at 20°C.

The role of the substituent on the 2′-position of the pentose is less obvious. From Fig. 7 it appears that a 2′-OMe leads to an increase in base stacking with respect to a 2′-OH and 2′-H. An examination of corresponding mononucleoside diphosphates containing cytosine residues apparently demonstrates only a negligible difference of the 2′-OMe as compared to 2′-OH at 26°C (Warshaw & Cantor, 1970). However, the temperature-dependence of the optical rotation at 268 nm for r(CmpC) was steeper than for r(CpC), so that the two curves (which the authors regard as being similar) intersect at 37°C; this may be taken to imply that the stabilizing effect of the 2′-OMe is lower than that of the 2′-OH above 37°C. It is unfortunate that the foregoing data were based on ORD measurements, which renders a strict comparison with other data somewhat difficult. More significant are the observations of Adler, Grossman & Fasman (1968), who showed that the B_{2u} band intensity of d(CpC) is much lower than that of r(CpC) and, as we may infer from the data of Warshaw & Cantor (1970), of r(CmpC).

The situation for dinucleoside monophosphates with adenine residues contrasts sharply with those containing uracil or cytosine residues. The appropriate data, taken from Maurizot, Brahms & Eckstein (1969) and Bobst et al. (1969b) are combined graphically in Fig. 8. Most striking is the fact that the intensity of the long-wavelength CD band is lower for the 2′-OMe than for the other two at all temperatures. But it will be noted that the temperature-dependencies for r(ApA) and d(ApA) intersect at about 40°C, so that above this temperature the degree of base stacking in r(ApA) is lower than for d(ApA).

We shall revert again, below, to the influence of the 2′-substituent on the degree of base stacking in connection with the analogous situation for polynucleotides.

A further point pertains to the widely discussed differences between the CD spectra of monomers and dinucleotides. From Fig. 1 it is clear that the spectra of the dinucleoside monophosphates embrace all the bands found in the monomers.

The intensities are altered, and there are small variations in the band locations; we therefore see no need to interpret these in terms of exciton splitting (Brahms & Brahms, 1970). The modifications in going from a monomer to a dinucleotide may result purely from differences in the alteration of intensities of the individual bands due to modifications in the neighbourhood of the chromophores.

Comment is also called for regarding the spectral differences between dU and dT on the one hand, and d(UpU) and d(TpT) on the other. From what was said, above, bearing in mind the differences between the monomers, one should expect the spectra of the dinucleoside monophosphates to differ appreciably. A reasonable explanation
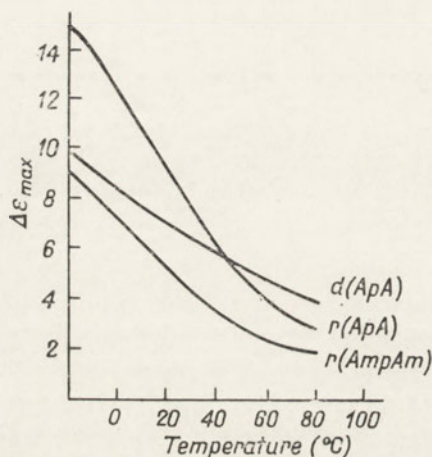

Fig. 8. Temperature dependence of intensities of long-wavelength positive (B_{2u}) bands of r(ApA) and r(AmpAm), taken from Bobst et al. (1969a); and d(ApA), taken from Maurizot et al. (1969).

is that the apolar 5-methyl substituent increases the degree of stacking in d(TpT) as compared to d(UpU). Increased interaction between bases might be expected to lead to conformational changes of the monomeric units in a dinucleotide; Emerson et al. (1967), in fact, showed a threefold increase in the Cotton effect of thymidine on transfer from water to the less polar dimethylformamide, indicative of a conformational change.

Single-stranded forms of polynucleotides. The spectra for the single-stranded forms of the polynucleotides are quantitatively similar to those for the corresponding dinucleoside monophosphates (Fig. 1), but they differ with regard to the temperature-dependence of the B_{2u} band intensities (Fig. 4). The band intensity for poly Um is higher than that for poly rU and poly dU, but the temperature-B_{2u} band intensity curves for the latter two intersect at 50°C. Similarly the curves for poly rT and poly dT intersect, at about 20°C.

This latter fact may be correlated with the observed temperature-dependence of quantum yield for photodimerization of thymine residues, which is presumably dependent on the degree of base stacking. For single-stranded poly rT and poly dT the quantum yields at 16°C are, respectively, 5.7 and 3.4 (in about 0.005 m-NaCl) and at 40°C 2.2 and 2.4 (Tramer, personal communication; Tramer et al., 1969). These results are qualitatively in accord with the CD data: at 16°C the B_{2u} band intensity is higher for poly rT, while at 40°C it is higher for poly dT.

From the foregoing it follows that the introduction of a 2′-OH in poly dU (or poly dT) leads to an increase in the degree of base stacking only over a defined temperature range. At higher temperatures the presence of the 2′-OH results in decreased stacking relative to the deoxy polymer. This behaviour differs from that prevailing for d(UpU) and r(UpU). Similar comparative data for other homopolymers demonstrate that, in the case of cytosine (Ts'o, Rapaport & Bollum, 1966; Adler, Grossman & Fasman, 1967) and adenine (Adler *et al.*, 1969; Bush & Scheraga, 1969) polynucleotides, the 2′-OH increases stacking at all temperatures; but, for the corresponding dinucleoside monophosphates, this holds only for that with cytosine residues.

The influence of the 2′-OMe substituent in poly Um is similar to that in the corresponding r(UmpU). The B_{2u} band intensity is higher than for r(UpU) or d(UpU), as is also the slope of the temperature-dependence of the B_{2u} band intensity. Again, however, this does not appear to be a general rule. The CD spectrum of poly Am practically coincides with that for poly rA (Bobst, Cerutti & Rottman, 1969a), both for the negative and positive bands. In turn, the spectrum of poly Cm differs only slightly from that for poly rC (unpublished results). It follows that the role of the ribose 2′-substituent cannot be considered apart from the association tendency of the aromatic bases.

Ordered forms of polynucleotides. It is at once apparent from Fig. 5 that the transition from the single-stranded to the structured forms of poly rU, poly rT and poly Um is accompanied by pronounced modifications of the CD spectra. It is, on other hand, quite striking that the spectra for the ordered forms of all three polymers are, apart from differences in band intensities, similar, not only as regards what we may refer to as the B_{2u} band at about 265 nm, but also the negative band at about 245 nm and the second positive band at about 225 nm. It is, of course, obvious that the reason for the approximately 6 nm shift, to the red, of the bands for poly rT is due simply to the bathochromic shift of the absorption spectrum of the ribosethymine residues by the 5-methyl pyrimidine substituent. Apart from the differences in T_m values for these three polymers, and the differences in cations utilized for the production of all three structured forms at room temperature, there is nonetheless indirect evidence suggestive of conformational differences between them. For example, an examination of the polyamine-induced maximal ordered forms exhibited by poly rU and poly rT demonstrated that the former required one equivalent of a dimethyleneamine, as compared to a pentamethyleneamine for the latter (Szer, 1966), suggestive of a difference in pitch of the two helical forms.

It is conceivable that such a difference in helix pitch accounts for the difference in intensities of the B_{2u} bands of poly rU and poly rT. It is known that different samples of double-helical DNA exhibit similar CD spectra with a common long-wavelength positive band at 273 nm (Brahms & Mommaerts, 1964), and it was shown by Samejima & Yang (1965) that the intensity of this band is dependent on the GC content.

The relatively feeble Cotton effect exhibited by helical DNA, in relation to RNA or homopolymers, was interpreted by Brahms & Mommaerts (1964) as due to

the fact that the former consists of two antiparallel strands. This is in agreement with the pronounced Cotton effect of acid helical poly rA (Brahms, 1964) which consists of two strands with the same polarity (Rich, Davies, Crick & Watson, 1961). Other examples include poly d(A-T), which forms a "hairpin" structure in which the strands are antiparallel (Scheffler, Elson & Baldwin, 1968) and which exhibits a feeble Cotton effect (Ts'o *et al.*, 1966); and poly rA·poly rU, shown by Arnott, Fuller, Hodgson & Prutton (1968) to consist of antiparallel strands, and likewise with a small Cotton effect (Brahms, 1965).

The foregoing may now be examined in the light of the pronounced Cotton effects shown by the ordered structures of poly rU, poly Um and poly rT (Fig. 5), which may be considered as consisting of two strands with the same polarity and involving base-pairing between $N_{(3)}H$ and $C_{(4)}=O$ of a base in one strand with $C_{(4)}=O$ and $N_{(3)}H$ of a base in the "complementary" strand (Scheme 1a). Such a
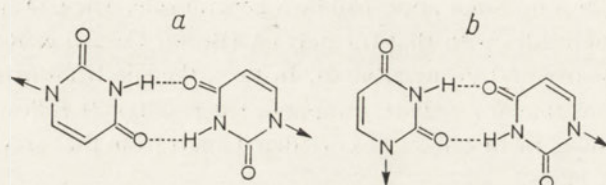
Scheme 1.

system of base-pairing is based on crystallographic data for 1-methyluracil and 1-methylthymine, although other possibilities likewise exist (Voet & Rich, 1970).

However, hydrodynamic studies on helical poly rU (Dourlent, Thrierr, Brun & Leng, 1970) have been interpreted in terms of a "hairpin" type structure. An examination with the aid of CPK models shows that, if we assume poly rU to be a twin-stranded helix in the form of a hairpin so that the two strands are anti-parallel, the *only* type of base-pairing possible is that between $C_{(4)}=O$ and $N_{(3)}H$ of a residue in one chain to $N_{(3)}H$ and $C_{(2)}=O$ in the complementary chain (Scheme 1b). Base pairing between $C_{(4)}=O$ and $N_{(3)}H$, and $N_{(3)}H$ and $C_{(4)}=O$ is possible only if one of the residues of each base-pair has the form *syn* with respect to the sugar, *or* if the polarization of the two strands is identical, i.e. the helical structure is not "hairpin" but is formed between two single strands[2].

Role of 2'-hydroxyl. For some time the differences between ribo and deoxyribo polymers were ascribed to a presumed intramolecular hydrogen bond between the 2'-hydroxyl and the nitrogenous base of the same residue (Ts'o *et al.*, 1966). With a view to clarifying the differences in optical properties of r(ApA) and d(ApA) Maurizot *et al.* (1969) proposed the existence of hydrogen bonding between the 2'-hydroxyl and the phosphate group at the 3'-position.

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[2] It should be noted that the situation is quite different when we are dealing with an alternating copolymer. For example, in the case of the alternating poly dAT, it is a simple matter to demonstrate with the aid of CPK models that a "hairpin" type of twin-stranded helix is possible, with Watson-Crick pairing of A-T base pairs.

However, studies on the properties of poly rU, poly rC and poly rA, referred to above, have since demonstrated that for all three of these the replacement of the 2'-hydroxyl by 2'-OMe not only does not affect the ability of these polymers to form ordered structures, but may even lead to an ordered structure with a higher stability than that of the corresponding ribo polymer. The logical conclusion which follows from this is that the 2'-hydroxyl is not essential as a hydrogen bond donor for formation of helical structures. It was consequently suggested that other factors, such as solvent effects, must be invoked.

In general, when one examines the deoxyribo, ribo and 2'-O-methoxyribo analogues of poly U, poly C and poly A, the most striking feature is the difference in behaviour of the deoxyribo analogues from the other two. An apparent exception to this rule is the behaviour of dinucleoside monophosphates of adenine (see above). But the most striking effect is undoubtedly the inability of poly dU (or poly dT) to form an ordered structure, even at low temperatures in the presence of polyamines.

From an examination of published data, and the results herein reported, it must be admitted that there is at present insufficient experimental evidence to evaluate unequivocally the nature of the factors responsible for the behaviour of a given model as a function of the substituent on the 2'-position of the pentose ring. The introduction of a 2'-OMe substituent in place of 2'-OH, while eliminating any possible role of the latter as a hydrogen bond donor, leads to the presence of an apolar group which may provoke an increase in stabilizing forces due to *hydrophobic interactions*, or conceivably to a decrease arising from steric hindrance.

Probably the simplest direct comparison possible is that between poly dU and poly rU (and, of course, poly dT and poly rT). The ability of the latter to form an ordered structure seems difficult to ascribe to the formation of hydrogen bonds involving the 2'-OH within each residue. However, one possibility which does not appear to have been considered is the formation of a hydrogen bond between the 2'-OH of one residue with the $O_{(4')}$ of the ribofuranose ring of the neighbouring residue as illustrated in Scheme 2 (Rabczenko & Shugar, 1970). A CPK model of poly rU
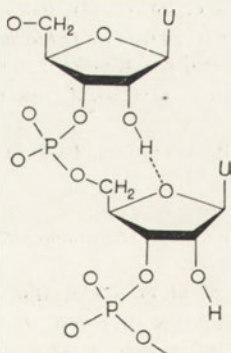
Scheme 2. Proposed scheme of hydrogen bonding of 2'-OH of one ribose residue in poly rU to $O_{(4')}$ of adjacent residue.

based on such a conception shows that the conformation angles in a model of this type are not in disagreement with accepted values. These values turn out to be $\Theta_1 = 260°$; $\Theta_2 = 240°$; $\Theta_3 = 260°$; $\psi = 90°$; $\varphi = 220°$; and they are all not far from the minimum potential on the curves representing the dependence of energy of formation of such a conformation on the angles (according to Lakshminarayanan & Sasisekharan, 1969b).

As far as we are aware, the only reasonable non-spectroscopic data regarding the conformation of the single-stranded forms of the foregoing polymers are those of Inners & Felsenfeld (1970) who employed hydrodynamic methods to show that poly rU exhibits a considerably greater degree of rigidity than that expected theoretically for a random coil. Formation of hydrogen bonds between the 2'-OH of one residue to $O_{(4')}$ of the adjacent one would readily account for this observed behaviour.

Furthermore, the synthesis of poly 2'-deoxy-2'-chlorouridylic acid has now been reported (Hobbs, Sternbach & Eckstein, 1971). This polymer was found to exhibit behaviour analogous to poly dU, i.e. is incapable of forming a helical structure. It exhibited no hyperchromicity on heating under various conditions, nor was it capable of forming a helical structure. The concept of a $2'$-OH$\cdots O_{(4')}$ hydrogen bond is in agreement with these observations since a 2'-Cl substituent, because of steric factors (the Van der Waals' radius of Cl is similar to that for OH), is unable to increase the hydrophobic interaction between bases.

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BADANIE KONFORMACJI NUKLEOZYDÓW, DWUNUKLEOZYDOMONOFOSFORA- NÓW I HOMOPOLINUKLEOTYDÓW ZAWIERAJĄCYCH URACYL LUB TYMINĘ ORAZ RYBOZĘ, DEZOKSYRYBOZĘ ALBO 2'-O-METYLORYBOZĘ

Streszczenie

Zarejestrowano serię widm kołowego dichroizmu (CD) w zakresie 220 - 330 nm dla szeregu pochodnych uracylu (lub tyminy) — nukleozydów, dwunukleozydomonofosforanów i homo-polinukleotydów w formie pojedynczej nici oraz w formie uporządkowanej, w których cukrem była ryboza, dezoksyryboza lub 2'-O-metyloryboza. Zmierzono również zależność temperaturową natężenia długofalowego, dodatniego pasma CD (B_{2u}) badanych związków. W celu sprawdzenia niektórych koncepcji dotyczących konformacji zbudowano modele cząsteczek z modeli atomowych Coreya-Paulinga-Koltuna (CPK).

Dla serii dwunukleozydomonofosforanów uracylu stwierdzono, że zarówno natężenie pasma B_{2u}, jak i nachylenie krzywej zależności temperaturowej tego natężenia rosną z temperaturą w ko-lejności: d(UpU), r(UpU), r(UmpU). Taka zależność świadczy o wzroście upakowania zasad w tej

samej kolejności, co jest zgodne z danymi literaturowymi na temat fotodimeryzacji d(UpU) i r(UpU).

Porównując dane CD dla poli dU i poli rU oraz poli dT i poli rT stwierdzono, że natężenie pasma B_{2u} jest większe dla rybopolimerów tylko poniżej pewnej temperatury. Wyniki te można wiązać z różnym upakowaniem zasad w różnych temperaturach, co jest również zgodne z danymi literaturowymi o fotodimeryzacji w funkcji temperatury dla poli dT i poli rT. Poli Um wykazuje w całym zakresie temperatur większe natężenie pasma B_{2u} niż inne polimery, co wskazuje na wyższy stopień upakowania zasad.

Uporządkowane formy poli rU, poli rT i poli Um mają uderzająco podobne widma CD różniące się jedynie natężeniami. Fakt ten tłumaczony jest podobieństwem struktur złożonych z dwóch nici o identycznej polarności.

Przedyskutowano rolę 2'-hydroksylu w rybopolinukleotydach. Porównanie danych literaturowych wskazuje, że wpływ na strukturę drugorzędową 2'-OH (lub 2'-H, czy 2'-O-Me) jest w dużej mierze zależny od rodzaju zasady azotowej w polimerze czy dwunukleozydomonofosforanie. Jednym z wniosków dyskusji jest postulat wiązania wodorowego pomiędzy 2'-OH jednej jednostki, a $O_{(4')}$ następnej. Taki model tłumaczyłby stwierdzoną większą sztywność poli rU w formie pojedynczej nici.

Addendum (added in proof). Since submission of the above manuscript, an interesting paper by Abraham (*J. Theor. Biol.* **30**, 83, 1971) has come to our attention. A new model for transfer ribonucleic acids is proposed, in which hydrogen bonding of the ribose 2'-OH hydroxyls to the $O_{(4')}$ of an adjacent ribose residue is considered as a possibility. We shall discuss this problem in detail in a forthcoming note (*Acta Biochim. Polon.* **19**, no. 1, 1972).

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CHEMICAL MUTAGENESIS: REACTION OF N-METHYLHYDROXYLAMINE WITH CYTOSINE ANALOGUES

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1. A study has been made of the reaction of N-methylhydroxylamine with cytosine, 5-methylcytosine and their glycosides with a view to clarifying the mechanism of N-methylhydroxylamine mutagenesis. 2. The product(s) of reaction of cytosine (and its glycosides) were shown to be capable of only a modified form of Watson-Crick G-C base-pairing. The implications with regard to the mechanism of hydroxylamine mutagenesis are discussed. 3. 5-Substituted cytosines (and their glycosides) do not react at all with N-methylhydroxylamine, because of steric hindrance due to the 5-substituent, thus readily explaining why this reagent is not mutagenic against T-even bacteriophages.

Hydroxylamine[1], methoxyamine and N-methylhydroxylamine together form a group of mutagens which are generally assumed to provoke genetic effects by a common chemical mechanism. The factor common to all three is their ability to react with cytosine (and 5- or 6-substituted cytosines) and cytosine glycosides. HA itself also reacts with uracil (but not thymine) to give a product which is non-mutagenic due to opening of the uracil ring (Shuster, 1961; Verwoerd, Kohlhage & Zillig, 1961); and with adenine, but at a *very* low rate (as compared to cytosine) (Budowsky, Sverdlov & Monastyrskaya, 1969). In view of the known action of HA in inducing only GC to AT transitions (Tessman, Poddar & Kumar, 1964; Brenner, Stretton & Kaplan 1965; Vanderbilt & Tessman, 1970) it appears unlikely that the reaction with adenine is of any significance.

There are, however, some well-defined differences between HA, OMeHA and NMeHA as regards their genetic effects. For example, a comparison of the mutagenic to inactivating effects on transforming DNA demonstrated that, at low concentrations (<1 M) the inactivation produced by HA and NMeHA (but not OMeHA) is considerably more pronounced than the mutagenic effects. This has been inter-

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[1] The following abbreviations are employed: HA, hydroxylamine; OMeHA, methoxyamine; NMeHA, N-methylhydroxylamine.

preted as due to secondary reactions deriving from the presence in HA and NMeHA of the -OH group, which leads to the production of free radicals. But, at higher concentrations ($\geqslant$1 M), the mutagenic effects of both these compounds exceed that of OMeHA (Freese & Freese, 1964; Freese, Gerson, Taber, Rhaese & Freese, 1967).

An additional, and more significant, difference between the foregoing derivatives is the apparent inability of NMeHA to induce mutations in phage T4 (Freese, Freese & Bautz, 1961), whereas both HA and OMeHA are mutagenic against the T-even bacteriophages (Freese et al., 1961; Champe & Benzer, 1962; Chubukov & Tatarinova, 1965).

The purpose of the present study is to attempt to clarify the nature of the reactions of NMeHA with cytosines and 5-substituted cytosines and to correlate these, insofar as possible, with the known mutagenic effects of NMeHA in in vitro systems.

MATERIALS AND METHODS

1-Methylcytosine was obtained by amination of 1-methyl-4-ethoxyuracil in methanolic ammonia (Schmidt-Nickles & Johnson, 1930). The HCl salt of NMeHA was an Aldrich (Milwaukee, Wis., U.S.A.) product. Cytosine, cytidine and 5-methylcytosine were purchased from Sigma (St. Louis, Mo., U.S.A.).

The products of reaction were studied by means of spectral methods, using a Unicam SP-500 instrument, and paper chromatography.

Spectral measurements of the reaction between NMeHA and cytosine analogues are complicated by the UV absorption of NMeHA extending as far as 270 nm, and moreover increasing with time. The following procedure was therefore adopted: the cytosine derivative was added to a neutral solution of NMeHA, and a portion of the latter alone served as a control, which was subjected to the same treatment as the mixture of NMeHA with the cytosine analogue. Following mixing of the two components, a portion was withdrawn at time 0, and the remainder transferred in 50 - 100 μl portions to test tubes which were then sealed. At various time intervals, one of the tubes was opened and a 1 μl sample added to 0.9 ml of 0.05 M-phosphate buffer, pH 7.5, for immediate recording of the UV spectrum, starting at the shorter wavelengths in order to minimize the effects of the time-dependent changes in the absorption of NMeHA. Simultaneously a sample was also taken for chromatography.

The following solvent systems were employed with ascending chromatography on Whatman paper no. 1: (A) water-saturated butanol; (B) ethanol - 1 M-ammonium acetate, 7:3, v/v; (C) ethanol - water, 4:1, using paper previously dipped in a solution of saturated ammonium sulphate - water, 1:9. As in the case of spectral measurements, the presence of NMeHA interfered with chromatographic identification of the reaction products. Furthermore NMeHA eluates, with R_F 0.14 in solvent A and 0.77 in solvent B, exhibited absorption maxima at about 225 nm, which could easily be mistaken for one of the reaction products. Consequently, paper chromatography made it possible to isolate only one of the reaction products, the R_F values of which are presented in Table 1.

Table 1

R_F *values of cytosine, 1-methylcytosine and cytidine, and their reaction products*
with NMeHA

Ascending paper chromatography using Whatman paper no. 1 and solvent systems *A*, *B* and *C*
(see text) were applied.

Compound	Solvent system		
	A	*B*	*C*
Cytosine	0.15	0.69	0.29
N^4-Methyl,N^4-hydroxycyto-sine	0.20	0.81	0.72
1-Methylcytosine	0.18	0.93	0.46
1,N^4-Dimethyl-N^4-hydroxy-cytosine	0.53	1.0	0.80
Cytidine	0.09	0.79	0.40
N^4-Methyl,N^4-hydroxycyti-dine	0.17	0.89	0.80

RESULTS

Reaction of NMeHA with cytosine and $N_{(1)}$*-substituted cytosine.* As in the case
of the reaction between HA and cytosine analogues (Verwoerd *et al.*, 1961; Brown &
Schell, 1961), two possible products were anticipated and sought for, illustrated by
II and III (Scheme 1). Compounds such as III had been previously prepared in crystal-

I

a) R = NHOH
b) R = NCH$_3$OH

II

a) R$_1$ = H   b) R$_1$ = CH$_3$
c) R$_1$ = ribose

III

Scheme 1

line form, IIIb by Brown, Hewlins & Schell (1968) and IIIa and IIIb by ourselves
(Janion & Shugar, in preparation). The lability of II if, in fact, it is formed at all,
has precluded its isolation but, by analogy with the corresponding products of
reaction of HA and OMeHA with cytosine (Brown & Schell, 1961, 1965; Janion &
Shugar, 1965b; Kotchetkov, Budowsky & Shibaeva, 1963), and, particularly, the
product of reduction or photohydration of N^4-semicarbazidocytosine (Janion &
Shugar, 1968) it would be expected to exhibit a λ_{max} at about 230 - 240 nm. Further-
more, in acid medium it should eliminate NMeHA from the 5,6 bond to give III.

The spectrum of N^4-methyl-N^4-hydroxycytosine is shown in Fig. 1a alongside that
of cytosine. Fig. 1b illustrates one of a number of experiments demonstrating the

changes in the spectrum of cytosine treated with NMeHA under the following conditions: 0.1 M-cytosine, 4 M-NMeHA, pH 6 (optimum for this reaction), at 37°C. An examination of the shapes of the curves, although pointing to the continuous formation of IIIa with time (see Fig. 1a), provides no clue as to whether II is present as a product of the reaction or not.
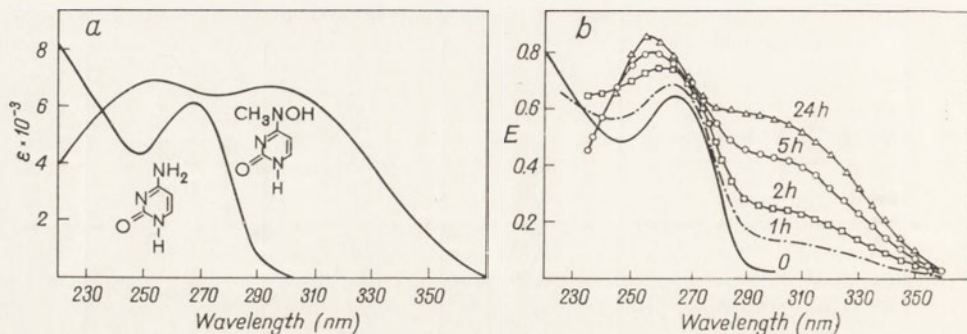

Fig. 1. *a*, Absorption spectra of cytosine and N^4-methyl-N^4-hydroxycytosine at pH 7. *b*, Modifications of absorption spectrum resulting from reaction of 0.1 M-cytosine with 4 M-N-methylhydroxylamine at pH 6 at 37°C.

The foregoing procedure was consequently modified as follows: The spectrum was run at a given time following the start of the reaction, then the solution was acidified with 10 µl conc. HCl to bring the pH to about 2, and then warmed to 85°C for 15 min[2]. This should result in transformation of any IIa formed to IIIa. The solution was then brought to neutrality and the spectrum recorded against a control solution of NMeHA which had been subjected to the same treatment. Figure 2 exhibits the results of one of several such experiments, the spectrum of the product(s) of reaction between cytosine and NMeHA prior to, and following, acid treatment.
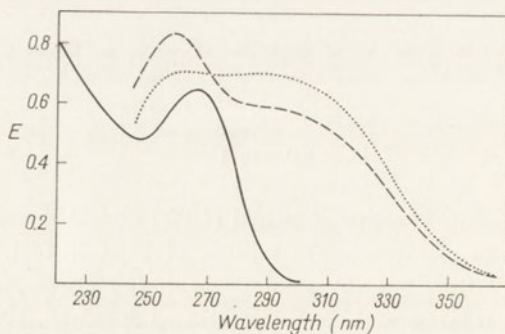
Fig. 2. Absorption spectra illustrating the reaction of 0.1 M-cytosine with 4 M-N-methylhydroxyl-amine at pH 6 and 37°C: ———, at time 0; — — —, after 24 h; · · ·, following acid treatment and neutralization. All spectra run at pH 7.4; see text for further details.

[2] We have since found that heating is not necessary for acid-catalysed conversion of II to III. This reaction goes to completion in 15 - 20 min at room temperature, further testifying to the lability of II.

The increase in absorption in the region 270 - 300 nm, resulting from the treatment of the reaction mixture with acid, clearly points to the existence in the primary reaction medium of a compound which is transformed by acid to N^4-methyl-N^4-hydroxycytosine (IIIa). This compound is presumably IIa. Furthermore, if we make use of the extinction coefficient for IIIa from Fig. 1a, the increase in absorption at about 300 nm in Fig. 2 can be used to calculate the amount of IIa formed under a given set of conditions.

Figures 3a and 3b exhibit the calculated ratios of IIa to IIIa with time during the course of the reaction of cytosine with NMeHA at pH 6 and 7. The reaction was
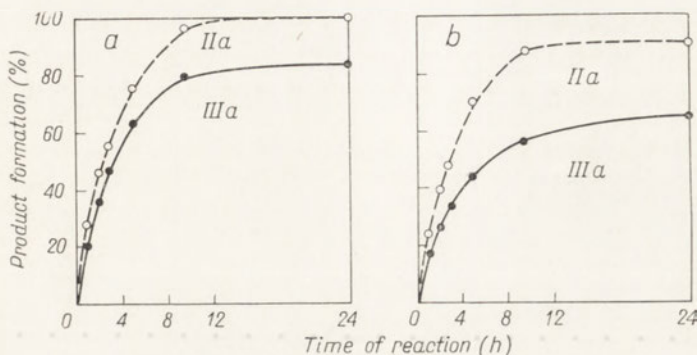
Fig. 3. Reaction rates, expressed in terms of percent formation of products IIc and IIIc, of 0.1 M-cytosine with 4 M-N-methylhydroxylamine at 37°C: *a*, at pH 6; *b*, at pH 7.

regarded as 100% complete when the optical density at 300 nm corresponded to the total conversion of cytosine to N^4-methyl-N^4-hydroxycytosine. The calculations made use of the molar extinction coefficients from Fig. 1a, for cytosine, 6.13×10^3 and 0.1×10^3 at 267 nm and 300 nm; and for IIIa, 6.6×10^3 at 300 nm.

A comparison of Figs. 3a and 3b shows that the pH of the reaction mixture influences (1) the reaction rate; at pH 6 the reaction is complete within 12 hours, whereas at pH 7 it is still incomplete after 24 hours; (2) the ratio of the products formed; at pH 6, the proportion of IIa to IIIa is about 16%, at pH 7 it is about 29%. The higher yield of IIa at pH 7 may be due either to a greater rate of formation of this compound, or to a decrease in its rate of transformation to IIIa.

An identical procedure was used to follow the reaction between 1-methylcytosine and NMeHA at pH 6 and 7, with overall findings exhibited in Fig. 4. Formation of IIb and IIIb in this case was observed by following the change in optical density at 300 nm after acid treatment, and calculated using the known molar extinction coefficients: for 1-methylcytosine, 8.15×10^3 at 274 nm and 0 at 300 nm; for 1,4-dimethyl-N^4-hydroxycytosine, 6.4×10^3 at 300 nm. The striking difference in behaviour of 1-methylcytosine with respect to cytosine, apart from the slower rate of reaction with NMeHA, is the formation at pH 6 of only IIb, whereas at pH 7 the proportion of IIb to IIIb is less than 5%.

The same procedure was applied once again, this time to the reaction of cytidine with NMeHA. In this instance the absence of the required reference compound,

N^4-methyl-N^4-hydroxycytidine, rendered quantitative measurements impossible. However, under no reaction conditions was there any indication from spectral measurements of IIc formation. Hence, if IIc is formed at all from the nucleoside (or from nucleotide residues in a polynucleotide), it apparently undergoes much more rapid transformation to IIIc than in the case of the free pyrimidine. It follows therefore that the mutagenic activity of NMeHA must be related to the formation of III in cytidine residues in nucleic acids.
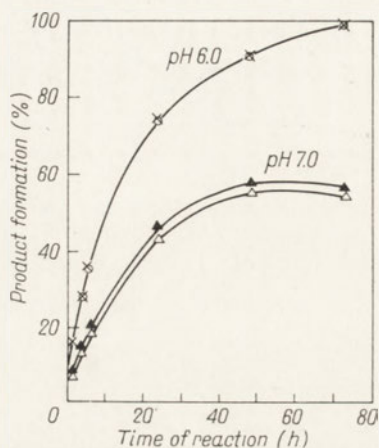
Fig. 4. Course of reaction be*ween 0.1 M-1-methylcytosine and 4 M-N-methylhydroxylamine, in terms of percentage of product IIIb formed, at 37°C; reaction run at pH 6: ○, prior to acid treatment; ×, following acid treatment and neutralization; reaction run at pH 7: △, prior to acid treatment; ▲, following acid treatment and neutralization.

The actual mechanism of reaction of NMeHA with cytosine is not clarified by the above. Is the product such as II formed by addition of NMeHA to III, or *via* addition of NMeHA to the cytosine ring, to give Ib, followed by nucleophilic replacement of the amino group by NMeHA to give II? In the case of the reaction of HA itself with cytosine, the presumed adduct of HA to the cytosine ring, Ia, (which is considered to facilitate subsequent nucleophilic replacement of the amino group by HA) is so unstable that it has not been possible to detect its presence by standard methods. Either it reverts to cytosine or to a derivative of type II. There is no reason to believe that the adduct of NMeHA to the 5,6 bond of the cytosine ring, i.e. Ib, would be any more stable than that of HA, i.e. Ia.

Reaction of NMeHA with 5-substituted cytosines. HA and OMeHA react with 5-substituted cytosine or cytosine nucleosides to give only one reaction product, analogues of III (Janion & Shugar, 1965a). It is generally accepted that the formation of this type of product leads to mutagenic effects of HA (and probably OMeHA) in the T-even bacteriophages (Janion & Shugar, 1965b; Phillips & Brown, 1967; Singer & Fraenkel-Conrat, 1969a).

An examination of the reaction of NMeHA (4 M, at pH 6) with 5-methylcytosine and 5-methyldeoxycytidine (0.02 M) demonstrated the complete absence of any

reaction product even after 100 hours at 37°C. No reaction could be observed also with derivatives of 4-ethoxy-5-methyluracil, in which the 4-ethoxy substituent is known to more readily undergo nucleophilic displacement by hydroxylamine (Janion & Shugar, 1965b). However, this lack of reactivity was not surprising in the light of numerous observations on the difficulties of introducing an exogenous dialkylamino substituent on the $C_{(4)}$ of a pyrimidine containing an alkyl substituent either at $N_{(3)}$ or $C_{(5)}$, due to steric hindrance of either of the latter (for review of this question, see Kulikowski, Żmudzka & Shugar, 1969). If any further argument in support of this were required, it is provided by the fact that 1,6-dimethyl-4-methoxypyrimidine readily reacts with NMeHA to give quantitatively 1,6,N^4-trimethyl-N^4-hydroxycytosine (Janion & Shugar, in preparation). It follows that the observed lack of mutagenic activity of NMeHA on T-even phages is due to the inactivity of this compound against the 5-hydroxymethylcytosine residues which replace cytosine in the T-even phage DNA.

DISCUSSION

The foregoing findings indicate that the principal, and probably only, stable product resulting from the reaction of NMeHA with cytosine derivatives is compound III, and that it is this product which presumably leads to mutations induced by NMeHA.

In accordance with the generally accepted theory regarding formation of mutations, the mutagenic properties of the hydroxylamine analogues of III have been interpreted in terms of the transition of this product to the imino form which, during replication, base pairs with adenine, as in Scheme 2.

N^4OH-cytosine   Adenine
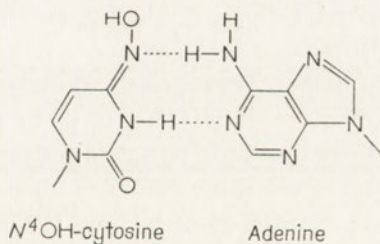
Scheme 2

It is, however, immediately obvious that this type of reasoning cannot interpret the mutagenic activity of a product such as III. The absence of a free hydrogen on the exogenous N^4 of III (or II, if we assume this is also active), does not permit of tautomerism to the imino form. Theoretically III (or II) could still undergo Watson-Crick base-pairing with guanine, but by formation of only two hydrogen bonds instead of three, and on the supposition that neither the N^4Me nor the N^4OH offer any steric hindrance to such base pairing. This is, of course, susceptible to direct test, and we have undertaken the preparation of the 1-cyclohexyluracil derivative of III with a view to examining its ability to base-pair with guanine in non-aqueous medium.

This dilemma was recognized by Phillips & Brown (1967), who proposed an alternative interpretation, viz. that the mutagenic species in this case is Ib, which, since it possesses a free amino group, can tautomerize to the imino form and base pair with uracil. However, in view of the established lability of the 5,6-HA adduct of cytosine (or cytidine), it appears most improbable that such a product could be involved in mutagenesis. The only argument in support of such a conception, viz. that the absence of mutagenic activity of NMeHA against T-even bacteriophages is due to the inability of the presumed 5-substituted derivative III to tautomerize (as a result of which only the NMeHA adduct to the 5,6 bond of cytosine i.e. Ib would be the mutagenic species), is obviously untenable in the light of the fact that NMeHA does not react with 5-substituted cytosine or its glycosides.

The foregoing is by no means the only illustration where mutagenic activity has been observed in the absence of any possibility of base pairing. For example methylation of cytosine on $N_{(3)}$ leads to a derivative which is apparently devoid of base-pairing ability. However, Ludlum & Wilhelm (1968) and Ludlum (1970) have shown that a copolymer of C and 3-MeC, when used as a matrix with RNA-dependent RNA polymerase, leads to the formation of a copolymer containing G and U residues.

Mutagenic effects resulting from the presence of 3-MeC have also been reported by Singer & Fraenkel-Conrat (1969b), who demonstrated a strict correlation between the 3-MeC content of TMV-RNA, following treatment with nitrosoguanidine, and the mutation frequency.

Furthermore, efforts to demonstrate directly the validity of the type of base-pairing exhibited in Scheme 2 have hitherto been negative. Neither homopolymers of N^4-hydroxycytidine, nor copolymers containing uridine residues, exhibited any ability to complex with poly A (Janion & Shugar, 1969).

Subsequently Smrt (1970) claimed to have observed such complex formation by allowing the mixtures of homopolymers to stand overnight, the argument being that the stability of these complexes is quite low so that longer periods are required to place interaction in evidence. We have repeated our previous experiments under the same conditions, and again noted the absence of complex formation. Furthermore, an examination of Fig. 8 of the paper by Smrt (1970) reveals a rather curious anomaly, viz. immediately after mixing the component homopolymers, the optical density of the mixture is greater than the theoretical sum for the components. This was not commented upon by the author, nor have we been able to observe it with our polymers. We consequently feel that our previous results are still valid, i.e. homopolymers of N^4-hydroxycytidine do not interact with the potentially complementary poly A.

It follows that the mechanism of mutagenic action of HA (or its methyl analogues) cannot be due to a simple change in base complementarity. Undoubtedly the modification in structure of a normal base (cytosine in this case) leads to a perturbation of the normal process of replication. But the ultimate effect of the presence of a modified base is probably intimately related to the specificity of the polymerase(s) involved.

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MUTAGENEZA CHEMICZNA: DZIAŁANIE N-METYLOHYDROKSYLAMINY NA ZWIĄZKI CYTOZYNY

Streszczenie

1. Dla wyjaśnienia mechanizmu mutagennego działania N-metylohydroksylaminy badano reakcję między N-metylohydroksylaminą a cytozyną i 5-metylocytozyną oraz ich glikozydami.

2. Struktura produktów reakcji umożliwia tylko modyfikację wytwarzanej zgodnie z teorią Watsona i Cricka pary zasad G-C. W świetle tych wyników i w powiązaniu z mechanizmem mutagennego działania hydroksylaminy przedyskutowano mutagenne właściwości N-metylohydroksylaminy.

3. 5-Podstawione związki cytozyny, ze względu na zawadę przestrzenną podstawnika 5, nie reagują w ogóle z N-metylohydroksylaminą, co tłumaczy dlaczego N-metylohydroksylamina nie powoduje mutacji u bakteriofagów parzystych.

J. T. KUŚMIEREK and D. SHUGAR

ALKYLATION OF CYTOSINE GLYCOSIDES IN ALKALINE MEDIUM: A NEW ROUTE TO THE PREPARATION OF O'-ALKYLATED NUCLEOSIDES AND NUCLEOTIDES OF CYTOSINE AND URACIL

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1. A study of the alkylation of 1-methylcytosine as a function of pH demonstrated that at pH values above 13 the ring $N_{(3)}$ nitrogen is inert to diazomethane and dimethylsulphate. 2. This observation was profited from to alkylate cytidine in alkaline medium to obtain a wide variety of O'-alkylated derivatives of cytidine. Deamination of these gave the corresponding O'-alkylated uridines. 3. The same procedures have been applied to obtain the 2'- and 3'-O-alkyl derivatives of cytidine-5'-phosphate. 4. The general applicability of this procedure is emphasized for the preparation of all possible O'-alkyl cytosine glycosides and, by deamination, the corresponding uracil glycosides.

It is widely and explicitly accepted that the ring $N_{(3)}$ nitrogen of 1-substituted cytosines is most susceptible to alkylating agents (Brookes & Lawley, 1962; Haines, Reese & Todd, 1964; Szer & Shugar, 1966). This position, and not the exocyclic amino group, is known to be the site of protonation in acid medium, testifying to the more nucleophilic character of the ring $N_{(3)}$ as compared to the exocyclic NH_2. It consequently proved surprising to us to find the ring $N_{(3)}$ quite inert to alkylating agents at pH > 13. As will now be shown, this fact makes possible relatively straightforward procedures for the preparation of pyrimidine nucleosides alkylated on the pentose moiety. Such nucleosides (and also nucleotides) are of interest in investigations on glycoside conformation, on dissociation of the sugar hydroxyls of nucleosides in alkaline medium (Fox & Shugar, 1952), and as potential antimetabolites. It should also be recalled that 2'-O-methylribose is fairly widely distributed in nature, particularly in tRNA and rRNA, while other O'-alkylated nucleosides are components of some antibiotics.

Treatment of 1-methylcytosine (I) with dimethylsulphate in the pH range 5 - 9, or with diazomethane in the pH range 5 - 12, was found to lead to formation of

1,3-dimethylcytosine (II). Further trials demonstrated that alkylation of I with di-methyl sulphate in more alkaline medium left the $N_{(3)}$ position unaffected and led to substitution of the amino group, with formation of IV and V. In 1 N-NaOH, with a 150-molar excess of dimethylsulphate, the products included 1 - 5% of IV; in 10 N-NaOH, under the same conditions, there appeared 10 - 20% of IV and se-veral percent of V. These products were identified by chromatographic and spectral methods. The reaction products also included traces of 1-methyluracil (III), but this was not due to the effect of the alkylating agent; the formation of trace amounts of deamination products of cytosine ribosides (up to 1 - 2%) in strongly alkaline medium (0.3 - 1.0 N-NaOH) has been previously reported by many investigators in connection with the widespread use of alkaline media for hydrolysis of RNA for analytical purposes.
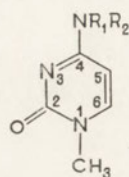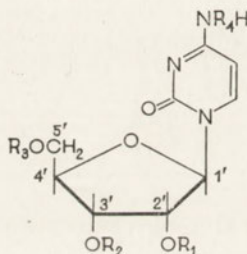

I $R_1 = R_2 = H$
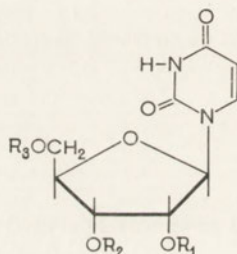IV $R_1 = H, R_2 = CH_3$
V $R_1 = R_2 = CH_3$
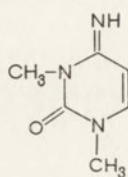
II
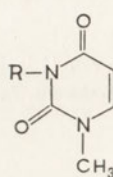
III $R = H$
VI $R = CH_3$

VII $R_1 = CH_3, R_2 = R_3 = R_4 = H$
IX $R_2 = CH_3, R_1 = R_3 = R_4 = H$
X $R_1 = R_2 = R_3 = R_4 = H$
XI $R_3 = CH_3, R_1 = R_2 = R_4 = H$
XII $R_1 = R_2 = R_3 = CH_3, R_4 = H$
XIII $R_1 = R_2 = R_3 = R_4 = CH_3$
XV $R_1 = R_2 = R_3 = C_2H_5, R_4 = H$
XVI $R_3 = trityl, R_1 = R_2 = R_4 = H$
XVII $R_1 + R_2 = isopropyl, R_3 = R_4 = H$

VIII $R_1 = H, R_2 = R_3 = trityl$
XIV $R_1 = R_2 = R_3 = CH_3$

The formation of IV and V cannot be interpreted in terms of an alkaline-cata-lysed (Dimroth) rearrangement of II as observed for other derivatives (Brookes & Lawley, 1960; Brown, 1961). At pH 12 - 13, 1,3-dimethylcytosine (II) is known to undergo uniquely deamination to 1,3-dimethyluracil (VI) (Brookes & Lawley, 1962). By contrast, 3-methylcytosine undergoes a rearrangement to N^4-methyl-

cytosine in a mixture of acetic anhydride, acetic acid and sodium acetate (Ueda & Fox, 1964). But the formation, under our totally different conditions, of an N^4,N^4-dimethyl derivative (V) fully excludes the possibility of such a rearrangement and suggests direct alkylation of the amino group. This is supported by the much earlier observations of Tschitschibabin, Konowalowa & Konowalowa (1921) and Tschitschibabin & Kunuianz (1928) that the sodium salt of 2-aminopyridine (the product of reaction of 2-aminopyridine with sodium amide), when treated with either methyl iodide or dimethylsulphate in anhydrous ether, yielded N^2-methylpyridine and N^2,N^2-dimethylpyridine; whereas reaction of 2-aminopyridine with methyl iodide (which served as solvent) gave only 1-methyl-2-aminopyridine. Direct alkylations of various other aromatic amines in alkaline medium, but under milder conditions (aqueous $NaHCO_3$) have been reported to yield products alkylated on the amino group in fairly high yields (Hünig, 1952).

A direct consequence of the foregoing is the possibility of the direct alkylation of the carbohydrate hydroxyls of cytosine nucleosides in alkaline medium under conditions where the aglycon is relatively inert.

2'-O-Methylcytidine (VII) was first obtained by methylation with methyl iodide of 3',5'-di-O-trityluridine (VIII) to give products alkylated on $N_{(3)}$, O^4 and $O_{(2')}$; the 2'-O-methyl-4-methoxy derivative, following ammonolysis and detritylation, yielded the desired VII (Furukawa, Kobayashi, Kanai & Honjo, 1965). Martin, Reese & Stephenson (1968) obtained VII and IX by direct methylation of cytidine (X) with diazomethane in aqueous dimethoxyethane; but the absence of $N_{(3)}$ methylation in this instance may have been due to the alkalinity of the reaction medium, since the dimethoxyethane solution of diazomethane was decanted from 40% KOH (without drying) directly into the aqueous solution of cytidine, so that the reaction medium must have been strongly alkaline.

We have found that alkylation of cytidine (X) with dimethylsulphate in 1 N-NaOH gave almost exclusively products alkylated on the ribose moiety. Paper chromatography of the reaction mixture (1 mmole of X, 100 ml 1 N-NaOH, and portionwise addition of 3×1 ml dimethylsulphate at room temperature with stirring) demonstrated, after 5 hours, starting material (46%, R_F 0.10), monomethylated product (37%, R_F 0.27), dimethylated (12%, R_F 0.48) and trimethylated (5%, R_F 0.69). Chromatography was ascending, with Whatman paper no. 3 and water-saturated n-butanol as solvent system. Elution with aqueous methanol of the mono-methylated fraction from a Dowex (OH^- form) 1×2 column (Gin & Dekker, 1968) showed that it consisted of a mixture of VII, IX and XI in the ratio 60:15:25. The R_F values for VII in various solvent systems agreed with those of an authentic sample of 2'-O-methylcytidine kindly made available by Dr C. B. Reese.

A chloroform extract of the reaction mixture (10 mmoles X, 100 ml 1.5 N-NaOH, 7×5 ml dimethylsulphate with addition of 10 ml 10 N-NaOH after each 5 ml portion of dimethylsulphate, following which the reaction mixture was left overnight at room temperature) contained XII, XIII and XIV in the ratio 80:15:5. The products were estimated by thin-layer chromatography on HF_{254} silica gel (Merck, Darmstadt, GFR) with chloroform-methanol (85:15, v/v), followed by elution and spectral

estimation. Three crystallizations from anhydrous ethanol provided chromatographically homogeneous 2′,3′,5′-tri-*O*-methylcytidine (XII), m.p. 239 - 241°C. An analogous reaction with diethylsulphate gave 2′,3′,5′-tri-*O*-ethylcytidine (XV), m.p. 165.5 - 166°C.

Deamination of XII with sodium bisulphite (Shapiro, Servis & Welcher, 1970; Hayatsu, Wataya & Kai, 1970) gave 2′,3′,5′-tri-*O*-methyluridine (XIV), m.p. 113.5 - 114.5°C. Treatment with dimethylsulphate of the alkali-stable 5′-tritylcytidine (XVI) and 2′,3′-*O*-isopropylidenecytidine (XVII) gave, respectively, 2′,3′-di-*O*-methylcytidine and 5′-*O*-methylcytidine. The use of diethylsulphate gave the corresponding ethylated derivatives. Deamination of all of these led to the corresponding uridine analogues. Apart from elementary analyses of a number of the foregoing, the structures were also confirmed by spectral analysis, periodate reaction, etc.

Special attention should be directed at this point to the 5′-*O*-methyl and 5′-*O*-ethyl derivatives of uridine, which have been used as starting products for the successful preparation of substrates for pancreatic ribonuclease which are fully resistant to phosphodiesterase II (Kole & Sierakowska, 1971; Kole, Sierakowska & Shugar, 1971).

The foregoing procedures have also been applied to the alkylation of cytidine-5′-phosphate to give products alkylated mainly on the 2′- and 3′-hydroxyls. Preparative paper chromatography (Whatman paper no. 3, ascending, with 5:2 (v/v) ethanol - 0.5 M-ammonium acetate) led to the isolation of a fraction in about 30% yield containing 90% 2′-*O*-methylcytidine-5′-phosphate and 10% 3′-*O*-methylcytidine-5′-phosphate. The composition of this fraction was determined by dephosphorylation with phosphatase to nucleosides and chromatography on a Dowex (OH⁻) column (Gin & Dekker, 1968). Attempts to separate the methylated cytidine-5′-phosphate isomers have hitherto proven unsuccessful.

The foregoing monomethylated fraction of cytidine-5′-phosphate was subjected to normal phosphorylation procedures to yield 2′(3′)-*O*-methylcytidine-5′-pyrophosphate which, under the conditions described by Janion, Żmudzka & Shugar (1970), readily polymerized to give what appears to be poly 2′-*O*-methylcytidylic acid.

Many of the above-mentioned new *O′*-alkylated derivatives of cytidine and uridine are now being employed in our laboratories for studies of the influence of *O′*-alkylation on the conformation of the ribose ring, the acidities of the ribose hydroxyls, the specificity of 2′(3′)-*O*-methylcytidine-5′-phosphate to 5′-nucleotidase, etc. Further details of the synthetic methods and properties of the products will be outlined elsewhere.

We should like to emphasize that the foregoing procedures should be applicable to the preparation of *O′*-alkylated derivatives of a wide variety of cytidine (and with the use of deamination procedures, the corresponding uridine) nucleosides and nucleotides, including also the deoxyribose analogues. The advantages of these procedures are perhaps best illustrated by the attempts of Codington, Cushley & Fox (1968) to prepare the 2′- and 3′-*O*-alkyl analogues of spongouridine (1-β-D-arabinosyluracil). The desired products were obtained, as might have been anti-

cipated, with the $N_{(3)}$ of the uracil ring also methylated. It is clear that application of our procedure to methylation of 1-β-D-arabinosylcytosine would lead to the mono-O'-alkyl derivatives of this important antimetabolite, and that subsequent deamination would give the desired mono-O'-alkyl derivatives of spongouridine. These preparations are now being undertaken in this laboratory.

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ALKILOWANIE GLIKOZYDÓW CYTOZYNY W ŚRODOWISKU ALKALICZNYM: NOWA DROGA OTRZYMYWANIA O'-ALKILOWANYCH NUKLEOZYDÓW I NUKLEOTYDÓW CYTOZYNY I URACYLU

Streszczenie

1. Badania nad alkilowaniem 1-metylocytozyny w zależności od pH wykazały, że w pH powyżej 13 atom azotu $N_{(3)}$ nie ulega metylowaniu dwuazometanem i siarczanem dwumetylu.

2. Otrzymano różne O'-alkilowane pochodne cytydyny przez alkilowanie cytydyny w środowisku alkalicznym. Dezaminacja tych pochodnych pozwoliła na uzyskanie odpowiednich O'-alkilowanych pochodnych urydyny.

3. Tę samą procedurę zastosowano do otrzymywania 2′- i 3′-O-alkilopochodnych cytydyno-5′-fosforanu.

4. Podkreślono ogólne znaczenie tej metody w otrzymywaniu wszystkich O′-alkiloglikozydów cytozyny, a przez dezaminację — odpowiadających im glikozydów uracylu.

Addendum (added in proof): Since submission of the above, we have applied the same procedure to alkylation of the sugar hydroxyls of arabinosylcytosine, with qualitatively similar results. Apart from the foregoing, 5′-O-methylcytidine has been converted to 5′-O-methylarabinosylcytosine *via* the intermediate 2,2′-anhydro-5′-O--methylcytidine, according to Kanai, Kojima, Maruyama & Ichino (*Chem. Pharm. Bull., Tokyo* **18**, 2569, 1970), a procedure we are now applying to the preparation of 3′-O-methylarabinosylcytosine. Meanwhile two additional procedures, applicable to the methylation of the 2′- and 3′-hydroxyls, have been described by Robins & Naik (*Biochemistry* **10**, 3591, 1971; *Biochim. Biophys. Acta* **246**, 341, 1971).

ELŻBIETA DZIEMBOR, J. GRYSZKIEWICZ and W. OSTROWSKI

PROPERTIES OF NEURAMINIDASE-TREATED ACID PHOSPHATASE OF PROSTATE

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The occurrence of isoenzymic forms of acid phosphatase from human prostate was found to be due to the content in the enzyme molecule of different amounts of *N*-acetylneuraminic acid.

Treatment with neuraminidase of the isolated fractions *EI* and *EII* resulted in formation of molecular species with identical isoelectric point (about pH 6.0), identical chromatographic pattern on DEAE-cellulose, and Stoke's radii which were smaller by about 2 Å than those for the native species.

The K_m values of *EI* and *EII* for *p*-nitrophenylphosphate, 5′AMP, 2′-+3′-CMP and "core" yeast RNA were of the same order of magnitude, although fraction *EII* showed somewhat higher substrate affinity than *EI*, and the neuraminidase-treated fractions had higher affinity than the native ones.

Citrate ion protected more efficiently the neuraminidase-treated fractions against fluoride inhibition. The reaction velocity with iodine monochloride was greater with the neuraminidase-treated fractions.

Human prostate, as demonstrated by Ostrowski & Rybarska (1965), contains two acid phosphatases (EC 3.1.3.2) which differ in their elution pattern from DEAE-cellulose as well as in electrophoretic mobility. This heterogeneity is due to the presence in the protein molecule of different amounts of NANA[1], which can be partly split off by neuraminidase from *Clostridium perfringens* (Smith & Whitby, 1968). Treatment of the phosphatases with neuraminidase from *Vibrio cholerae* abolishes their electrophoretic heterogeneity and alters some of their properties, e.g. the isoelectric point and substrate affinity (Ostrowski, Wasyl, Weber, Gumińska & Luchter, 1970; Dziembor, Gryszkiewicz & Ostrowski, 1970).

The present paper describes other physico-chemical and enzymic properties of the two acid phosphatases, which, after splitting off NANA, were found to possess similar, or identical, physico-chemical and enzymic properties.

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[1] Abbreviations: *EI*, *EII*, native phosphatase fractions; *EIn*, *EIIn*, neuraminidase-treated fractions; NANA, *N*-acetylneuraminic acid; pNPP, *p*-nitrophenylphosphate.

MATERIAL AND METHODS

Human hypertrophic prostate glands were obtained from the Urological Clinics in Cracow and Lublin. Tissues removed at operation were frozen and stored at -25°C.

DEAE-cellulose was purchased from Serva Co. (Heidelberg, West Germany). Sephadex G-100 and G-200, medium and superfine, were products of Pharmacia (Uppsala, Sweden). Carrier ampholytes (Ampholine), batches nos. 26 and 27, with pH range 3 - 6, 4 - 6 and 4 - 7 were products of LKB (Stockholm, Sweden). 5′-AMP, pNPP (disodium salt), NANA and chymotrypsin were from Koch-Light (Colnbrook, Bucks., England). Neuraminidase from *Cl. perfringens* was a product of Worthington Biochem. Co. (Freehold, N. J., U.S.A.). "Core" from yeast RNA was prepared by the method of Markham & Smith (1952). The mixture of 2′-+3′-CMP was from Boehringer (Mannheim, West Germany). Cytochrome *c*, from bovine heart, was a product of Biomed (Kraków, Poland); pepsin was from Nutritional Biochem. Co. (Cleveland, Ohio, U.S.A.); bovine serum albumin was from British Drug Houses (Poole, Dorset, England); γ-globulin was from Wytwórnia Surowic i Szczepionek (Warszawa, Poland); alcohol dehydrogenase was from K. & K. Lab. (Long Island, N.Y., U.S.A.); ovoalbumin was from CIECH (Gliwice, Poland). Acrylamide and N,N,N',N'-tetramethylenediamine (Temed) were obtained from Eastman Kodak Co. (Rochester, N.Y., U.S.A.). All other reagents were of analytical grade.

Isolation of fractions EI *and* EII *of acid phosphatase.* The isolation was carried out at 4°C. Pure phosphatase fractions were isolated from 470 g of pooled prostate glands which were collected and stored during about three months. Fraction *EI* was obtained as described by Ostrowski (1968). Fraction *EII* was purified as follows. Active fractions of enzyme *EII* obtained by separation on a DEAE-cellulose column (Ostrowski *et al.*, 1970) were dialysed against 0.0175 M-Na-phosphate buffer of pH 7.0, and rechromatographed on a DEAE-cellulose column (40×3 cm) equilibrated with the same buffer. About 150 ml of a solution (the activity of which corresponded to 0.2 μmole of pNPP split/ml/min) was applied to the column and after washing with 300 ml of the starting buffer, the enzyme was eluted with a concave gradient from 0.0175 M-Na-phosphate buffer of pH 7.0 to 0.1 M-Na-phosphate buffer of pH 6.0. Phosphatase *EII* emerged from the column when about 0.045 M concentration of buffer and pH 6.4 were attained. Fractions with the highest specific activity were combined, dialysed against 0.5 M-KCl - 0.05 M-tris-HCl buffer of pH 6.5, concentrated, applied to a Sephadex G-100 column (120×1 cm) and eluted with the same buffer. Fractions with constant specific activity were frozen at -25°C and used in further experiments. The yield was on the average 43% of the total activity of the fraction obtained after the first DEAE-cellulose column chromatography. Mean specific activity of phosphatase *EII* was 1.5 - 1.7 mmoles of hydrolysed pNPP/mg of protein/min, and was somewhat lower than the specific activity of phosphatase *EI* which was usually 1.7 mmoles/mg/min. When examined by disk electrophoresis at pH 8.5 and 3.6, the *EII* preparation gave one sharp band coinci-

ding with enzyme activity, and a weak additional band of inactive protein, about 5% of the total, migrating faster to the anode.

Estimation of acid phosphatase activity. This was determined with 0.01 M-pNPP solution as substrate in 0.2 M-citrate buffer of pH 5.0 at 37°C (Ostrowski & Tsugita, 1961). The enzyme activity was expressed in μmoles of *p*-nitrophenol liberated by 1 ml of enzyme solution during 1 min, or calculated per 1 mg of protein.

K_m and K_i values were determined graphically according to Lineweaver & Burk (1934). As substrate, pNPP, 5'-AMP, a mixture of 2'- and 3'-CMP, and "core" from yeast RNA were used. Appropriate concentrations of the substrates were prepared in 0.1 M-citrate buffer of pH 5.5. Enzyme activity was expressed in μmoles of liberated P_i, determined by the method of Fiske & Subbarow in the modification of Martland & Robison (1926).

Incubation of phosphatase with neuraminidase and quantitative determination of NANA. Samples of phosphatase *EI* or *EII* containing 0.2 - 0.5 mg of protein in 1 ml of 0.1 M-acetate buffer of pH 5.0 were incubated with 2 μg of neuraminidase from *Cl. perfringens* (0.002 unit) at 20°C for 20 h, and then dialysed overnight against this buffer which was to be used in further experiments. Under these conditions, about 80% of NANA present in the enzyme was liberated; as demonstrated by Ostrowski *et al.* (1970), the remaining 20% cannot be split off by neuraminidase.

Total content of NANA bound with protein was determined after hydrolysis in 5% trichloroacetic acid solution on a boiling water bath for 15 min (Molnar, Robinson & Winzler, 1965), and the liberated NANA was assayed by the thiobarbiturate method described by Warren (1959).

Electrophoretic separations. Disk electrophoresis was performed on 7.5% polyacrylamide gel under conditions described by Ostrowski *et al.* (1970). Tris-HCl buffer of pH 8.5 and acetate buffer of pH 3.6, both of ionic strength 0.025, were used. Acid phosphatase was localized on the basis of the colour reaction with *a*-naphtholphosphate as substrate (Allen & Gockerman, 1964). After electrophoretic separation, the gel rod was incubated for 20 min at 37°C in 10 ml of 0.02 M-citrate buffer of pH 5.5 containing 1 tablet of *a*-naphtholphosphate (Gödecke A. G., Freiburg, West Germany) and 10 mg of solid diazonium salt Fast Red B (Serva, Heidelberg, West Germany). After development of red colour in the zones containing the enzyme, it was stabilized by immersing the gel for 15 min in 7% acetic acid solution containing 0.1 M-ZnCl$_2$.

Isoelectric focusing was performed in the apparatus and under conditions described by Ostrowski *et al.* (1970).

Determination of Stoke's radius. The effective radius of the molecule of native and neuraminidase-treated *EI* and *EII* was determined by Sephadex G-200 filtration, on a column as described by Ostrowski & Wasyl (1969) and by the thin-layer technique (Radola, 1968). The column and plate were calibrated using standard proteins of known molecular weight and known Stoke's radii. The elution volumes of the proteins applied to the column were determined at 280 nm on a Uvispec (Hilger-Watts, London, England) spectrophotometer, and the elution volume of phosphatase was determined with pNPP as substrate. On thin-layer filtration, phosphatase activity

was localized in the gel layer by means of the filter paper print technique under the same conditions as in disk electrophoresis. The proteins were localized with Amido Black 10 B.

Other methods. Protein in chromatographic and electrophoretic fractions was estimated by measuring the extinction at 280 nm. For other purposes, protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951). pH measurements were made on a Ridan pH-meter (Unipan, Warsaw, Poland) with glass electrode type SLb-22, giving readings with an accuracy to ± 0.005 unit.

RESULTS

Content of NANA. Quantitative analysis of the content of NANA in fractions *EI* and *EII* is shown in Table 1. The results are calculated in moles of NANA per 100 000 g of protein, as this value was found to be the molecular weight of prostatic phosphatase (Derechin, Ostrowski, Gałka & Barnard, 1971). In all cases the content of NANA in fraction *EII* was higher by about 30% than in *EI*. There were quite large differences between the particular preparations, due probably to normal, individual variation of the NANA content in the enzyme. Also the influence of conditions and time of storage of prostate glands before enzyme extraction cannot be excluded.

Table 1

Content of NANA in acid phosphatase fractions

The numbers are moles of NANA per mole of phosphatase protein and represent mean values of three to six individual determinations.

Preparation	Phosphatase fraction	
	EI	EII
1	4.9±0.6	5.1±0.7
2	7.6±1.1	—
3	5.3±0.7	—
4	7.1±1.2	11.3±1.2
5	6.5±0.9	8.8±1.0
6	7.5±0.9	14.0±1.2
7	—	10.5±1.1
8	—	8.5±0.8
Mean	6.4±0.9	9.3±1.0

Electrophoretic mobility. The mixture of pure fractions *EI* and *EII* was separated by disk electrophoresis at pH 3.6 and 8.5 into two active bands. On the other hand, the mixture of fractions treated with neuraminidase gave only one active band with lower anodic mobility (Fig. 1).

In isoelectric focusing, when separation was accomplished in different pH gradients, either fraction of native enzyme separated into 3 - 4 subfractions with isoelectric points between about pH 4 and 5.2. A typical separation is shown in Fig. 2. During electrolysis in the presence of Ampholine, phosphatase underwent denaturation and a deposit of inactive protein was focused in the most acidic area of the

gradient column. Similar interaction was observed with other proteins (Pajandier, Audran & Steinbach, 1971). Neuraminidase-treated phosphatases *EI* and *EII* lost their heterogeneity, exhibiting a single, symmetrical active peak with isoelectric point at pH about 6.0. These results are in agreement with our previous observations
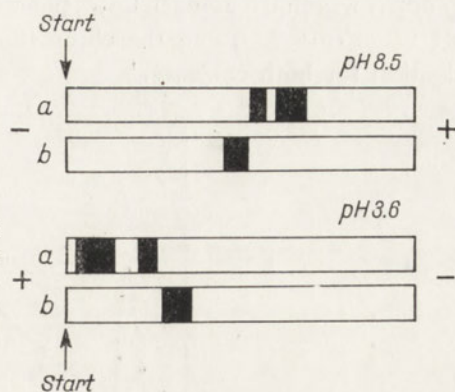

Fig. 1. Diagram of the disk electrophoresis patterns of the mixture of pure acid phosphatase fractions, *EI* and *EII*: *a*, native; *b*, neuraminidase-treated fractions.
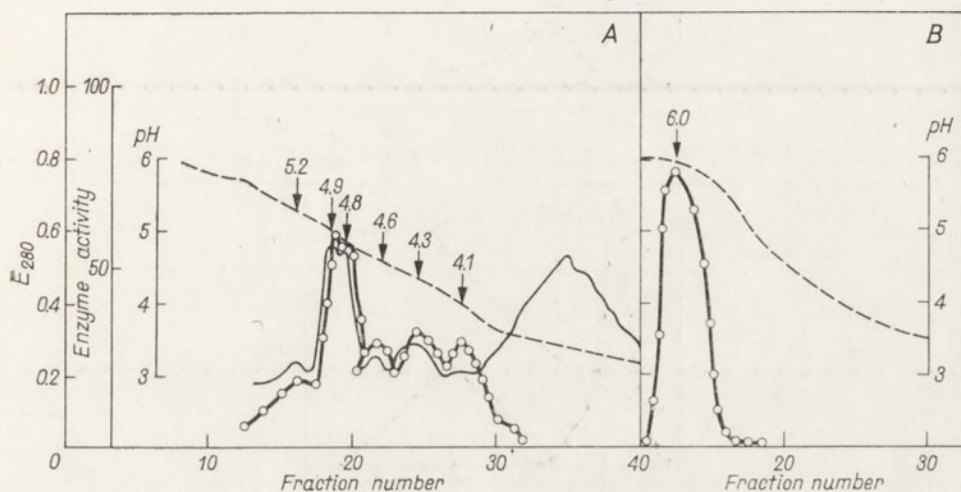

Fig. 2. Isoelectric focusing of *A*, native, and *B*, neuraminidase-treated phosphatase fractions. Picture *A* is a diagram composed of two independent separations carried out for enzyme *EI* (pH 6.0 to 4.0) and for enzyme *EII* (pH 6.0 to 3.0). Picture *B* is the result of separation (gradient between pH 4 to 7) of the mixture of neuraminidase-treated fractions *EI* and *EII*. ——, Protein at 280 nm after subtracting the average baseline extinction of the Ampholine; ○, enzymic activity; — — —, pH gradient. The detailed procedure for isoelectric focusing is given in Ostrowski *et al.* (1970).

(Ostrowski *et al.*, 1970) indicating that the presence of different amounts of NANA is the main cause of the electrophoretic heterogeneity of the acid phosphatase from prostate.

Adsorption properties. Equal portions of phosphatase *EI* or *EII*, treated and untreated with neuraminidase, were adsorbed on a small column of DEAE-cellulose equilibrated with 0.01 M-Na-phosphate buffer of pH 8.0, and then eluted with a continuous pH and phosphate concentration gradient (Fig. 3). *EI* was eluted at

pH 6.8 and 0.026 M buffer concentration whereas *EII* was eluted at pH 6.4 and 0.04 M. Under the described conditions of chromatographic separation, both fractions showed non-symmetrical peaks, confirming their heterogeneity demonstrated by isoelectric focusing. On the other hand, the whole amount of neuraminidase-treated *EI* or *EII* was eluted at much lower phosphate concentration (0.015 M) and at a higher pH value (pH 7.8), and the chromatographic patterns became symmetrical and identical for both enzymes.
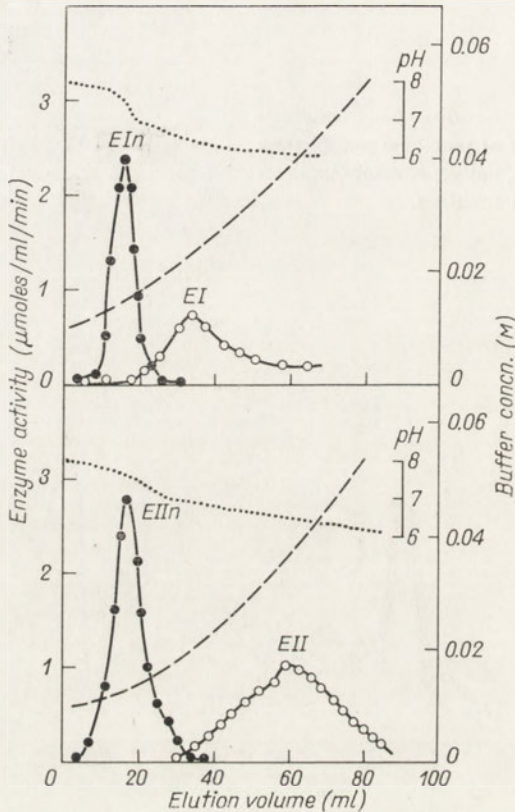

Fig. 3. Elution patterns of acid phosphatase fractions, *EI*, *EIn* (top) and *EII*, *EIIn* (bottom). Each diagram is composed of two independent separations of ○, native, and ●, neuraminidase-treated fractions. The sample (about 0.2 mg of protein in 1.5 ml) was applied on DEAE-cellulose column (10 × 1 cm) equilibrated with 0.01 M-phosphate buffer, pH 8.0. The enzyme was eluted with a concave gradient from 0.01 M, pH 8.0, to 0.1 M-phosphate buffer, pH 6.0. Fractions of 2 ml were collected at a rate of 8 ml/h at 4°C. ···, pH gradient; — — —, buffer concentration gradient; protein was not determined.

A similar result was obtained when a mixture of both enzyme fractions, treated and non-treated with neuraminidase, was submitted to DEAE-cellulose column chromatography (Fig. 4). This shows that the desialophosphatase could be readily separated from the fractions of the native enzyme by a single chromatographic procedure.
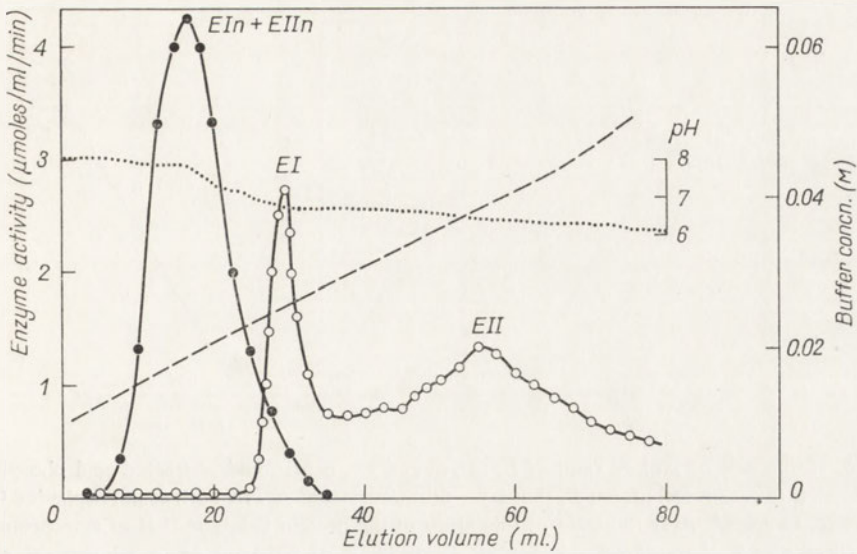
Fig. 4. Elution patterns from DEAE-cellulose of the mixture of ○, native, and ●, neuraminidase-treated acid phosphatase fractions. Conditions as in Fig. 3.

Determination of Stoke's radius. Since the phosphatase fractions *EI* and *EII* on Sephadex G-100 or G-200 gel filtration had the same elution volume (Ostrowski & Wasyl, 1969; Ostrowski *et al.*, 1970), a mixture of both fractions was used for the determination of Stoke's radius. The elution volumes of native and neuraminidase-treated phosphatases from Sephadex G-200 (column and thin-layer techniques) were compared with those of a number of standard proteins. The correlation between the elution volume and Stoke's radius for the proteins studied is shown in Fig. 5. By both techniques, the mean Stoke's radius of native phosphatase was 47.5 Å, in agreement with our previous results (Ostrowski & Wasyl, 1969), whereas the values for the enzyme treated with neuraminidase were 45 - 46 Å (mean 45.7 Å). This decrease in Stoke's radius indicates the occurrence of conformational changes in the molecule after splitting off NANA, leading to more compact structure. Similar results were obtained with other sialoproteins (Gottschalk & Thomas, 1961).

Effect of neuraminidase treatment on enzymic activity. As demonstrated by Ostrowski *et al.* (1970), after 24 h incubation of prostatic phosphatase with neuraminidase, about 80% of NANA was liberated but the enzymic activity was not appreciably altered. However, the fact that splitting off NANA resulted in changes of the isoelectric point, adsorption on DEAE-cellulose and molecular radius (see above) pointed to the possibility of subtle changes occurring also in the enzyme kinetics. This was studied and preliminary information has been reported by Dziembor *et al.* (1970).

Effect on optimum pH. Experiments were performed in 0.1 M-citrate and 0.1 M-tris-maleate-HCl buffer. The optimum pH value for *EI* and *EII* both for pNPP and 5′-AMP as substrate was found to be pH 5.5. After treatment with neuraminidase,
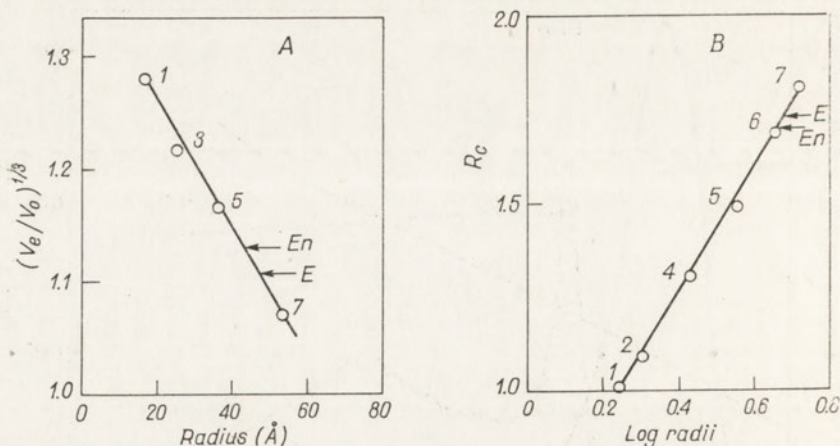
Fig. 5. Estimation of Stoke's radii of *E*, native and *En*, neuraminidase-treated acid phosphatase. *A*, Column filtration technique; *B*, thin-layer filtration technique. In both cases, Sephadex G-200, superfine, was used. R_c is the ratio of migration of the protein bands to that of cytochrome *c*. *1*, Cytochrome *c*; 2, chymotrypsin; 3, pepsin; 4, ovoalbumin; 5, bovine serum albumin; 6, alcohol dehydrogenase; 7, γ-globulin.

the optimum pH changed only slightly being increased by 0.3 pH unit in the case of 5′-AMP, and lowered with pNPP as substrate.

Effect on Michaelis constant. K_m values for different substrates, calculated from the Lineweaver-Burk plots, were of the same order of magnitude for the native and neuraminidase-treated enzyme (Table 2). However, native *EII* showed a slightly higher affinity than *EI* toward all the substrates, and the neuraminidase-treated enzyme had higher V_{max} than the native one; fraction *EIIn* had greater affinity toward the substrates than *EIn*. The data presented in Table 2 also indicate that both fractions of acid phosphatase after splitting off NANA represent proteins with similar or

Table 2

K_m *values for native and neuraminidase-treated phosphatase fractions with different substrates*

Initial reaction velocities were determined in 0.1 M-citrate buffer, pH 5.5, after 10 min of incubation at 37°C. The substrate concentration ranged from 0.1 to 0.3 mM. To 0.8 ml of the reaction mixture, 1 μl of enzyme solution was added (spec. act. 2.4 μg pNPP/min/μl). The enzymic activity was determined and expressed as described in Material and Methods. The data represent average values from at least five determinations, using different enzyme preparations.

Substrate	EI		EII	
	native K_m (mM)	neuraminidase-treated K_m (mM)	native K_m (mM)	neuraminidase-treated K_m (mM)
pNPP	0.79	0.70	0.57	0.33
5′-AMP	1.9	1.0	1.5	0.99
(2′+3′)-CMP	0.68	0.50	0.61	0.44
"Core" of yeast RNA	6.2	5.0	5.0	2.0

identical enzymic properties. Their increased affinity for the substrates studied following treatment with neuraminidase is probably due to facilitation of formation of enzyme-substrate complex as a result of diminished surface charge.

Effect on K_i. Phosphatases *EI* and *EII* after treatment with neuraminidase were more strongly inhibited by L-tartrate, which was demonstrated by Abdul-Fadl & King (1949) to be a competitive inhibitor, than the native enzymes (Fig. 6). The
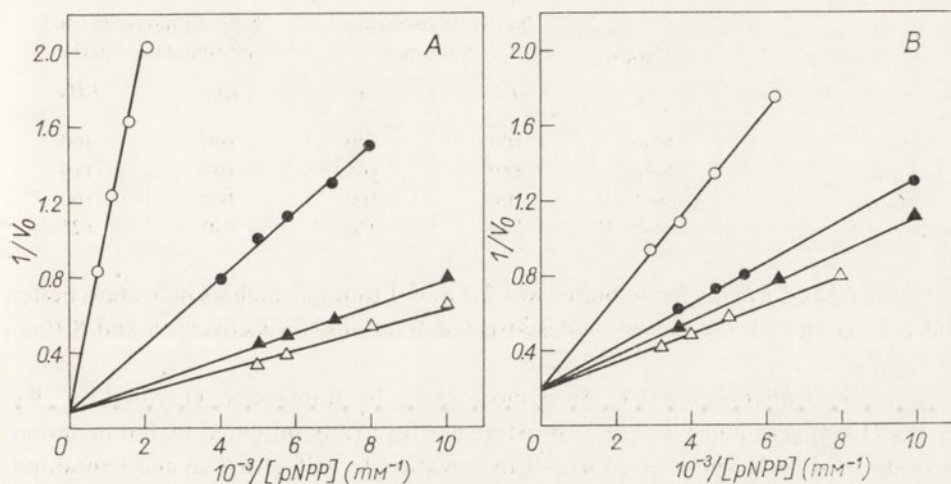

Fig. 6. Inhibition by L-tartrate of acid phosphatase fractions: *A*, *EI*, and *B*, *EII*, treated and untreated with neuraminidase. Each fraction (1 µl, cf. legend to Table 2) was incubated for 10 min at 37°C in 0.9 ml of 0.1 M-citrate buffer, pH 5.5, containing the indicated concentrations of pNPP as substrate. Inhibitor was added at 1 mM concentration. The enzymic activity was determined and expressed as described in Materials and Methods. Outlined symbols, neuraminidase-treated enzyme: ○, with inhibitor and △, without inhibitor; full symbols, untreated enzyme: ●, with inhibitor and ▲, without inhibitor.

K_i values for fractions *EI* and *EII* were for the native enzyme 0.077 and 0.062 mM, respectively, and after treatment with neuraminidase, 0.045 and 0.059 mM, respectively.

Effect on inhibition by F^-. Reiner, Tsuboi & Hudson (1955) have shown that fluoride is a competitive inhibitor of human prostate acid phosphatase, and that citrate and oxalate ions protect the enzyme against this inhibition. Since effectiveness of the protection by citrate ion depends on the electric state of the enzyme molecule (Anagnostopoulos, 1953), the inhibition of desialophosphatase by fluoride was studied in the presence and absence of citrate.

Appropriate enzyme preparations were incubated with 0.02 M-pNPP solution as substrate in the presence of 6 mM-NaF in 0.016 M-acetate or citrate buffer of pH 5.5. The effect of citrate was calculated as percentage of the activity in acetate buffer.

The results shown in Table 3 indicate that in the absence of fluoride each of the enzyme fractions studied had practically the same activity in citrate as in acetate buffer. On the other hand, in the presence of fluoride the activity of the native

Table 3

Protective effect of citrate buffer against fluoride inhibition of phosphatase fractions

The reaction mixture in 0.5 ml of 0.016 M buffer solution (acetate or citrate), pH 5.5, contained: pNPP (0.02 M) as substrate and, where indicated, NaF. After adding 1 μl of enzyme solution (spec. act. 2.4 μg pNPP/min/μl), the mixture was incubated at 37°C for 4 min. The results are expressed as percentages of the activity in acetate buffer (in the absence or presence of NaF, respectively) which was taken as 100%.

Buffer	Addition	Native phosphatase fraction		Neuraminidase-treated phosphatase fraction	
		EI	*EII*	*EIn*	*EIIn*
Acetate	None	100	100	100	100
Citrate	None	110	105	107	116
Acetate	6 mM-NaF	100	100	100	100
Citrate	6 mM-NaF	250	370	630	825

fractions *EI* and *EII* in citrate buffer was 2.5 and 4 times as high as in acetate buffer, and the activity of the neuraminidase-treated fractions, respectively, 6 and 8 times as high.

Effect of inhibition by ICl. As demonstrated by Bobrzecka, Ostrowski & Rybarska (1968) acid phosphatase of prostate is irreversibly inhibited by iodine monochloride at pH 8.1. The time-course of inactivation by ICl of native and neuraminidase-treated phosphatase is shown in Fig. 7. The incubation was carried out at 20°C with 0.03 mM-ICl in 0.05 M-tris-HCl of pH 8.1. The reaction of ICl with *EIn* was faster than with *EI*. This may be explained by assuming that changes in the conformation of acid phosphatase after treatment with neuraminidase lead to
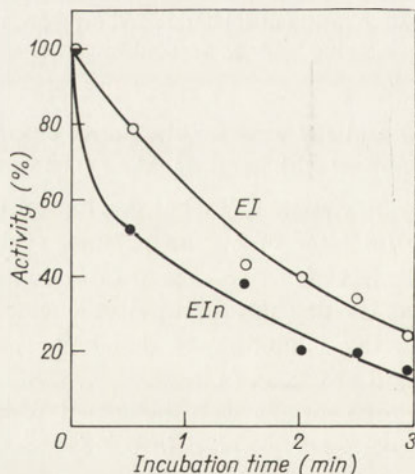

Fig. 7. Inactivation of ○, native, and ●, neuraminidase-treated phosphatase fraction *EI* by iodine monochloride. The enzyme (2 μg) in 100 μl of 0.05 M-tris-HCl buffer, pH 8.1, was incubated at 20°C with 0.03 mM-ICl. At the indicated times, the reaction was stopped by adding 200 μl of 30 mg% solution of crystalline bovine serum albumin and the residual enzymic activity was determined with pNPP as substrate.

uncovering of tyrosine and tryptophan residues and their increased reactivity with iodine — taking into account that both aromatic amino acids can be modified by this reagent (Bobrzecka *et al.*, 1968; Bobrzecka, Rybarska & Ostrowski, 1969).

DISCUSSION

The results of the present study confirm our previous observations (Ostrowski & Rybarska, 1965; Ostrowski *et al.*, 1970) that acid phosphatase in the hypertrophic human prostate occurs in more than one molecular form. On ultracentrifugation and filtration on dextran gel, i.e. with respect to molecular weight, the phosphatase appeared to be homogeneous (Ostrowski, 1968; Ostrowski *et al.*, 1970) but on DEAE-cellulose chromatography and disk electrophoresis it separated into two fractions, *EI* and *EII*. Isoelectric focusing (Ostrowski *et al.*, 1970) and electrophoresis on starch gel (Lundin & Allison, 1966; Smith & Whitby, 1968) revealed the heterogeneity of these two fractions, which separated each into a number of subfractions.

Phosphatase fractions *EI* and *EII* isolated in the pure state appeared to be sialo-proteins containing (in addition to other carbohydrates, as demonstrated by Derechin *et al.*, 1971), different amounts of NANA, 80% of which could be released by neuraminidase from *V. cholerae* or *Cl. perfringens*. After treatment with neuraminidase, both fractions had similar or identical physico-chemical properties, as shown by: identical elution pattern from DEAE-cellulose column, identical electrophoretic mobility in polyacrylamide gel, and identical isoelectric points as determined by isoelectric focusing.

Comparison of the activities of two native and neuraminidase-treated fractions of acid phosphatase indicates that although the hydrolysable NANA residues are not at the active site, their removal alters some of the kinetic properties of the enzyme, as shown by the K_m and K_i values for the native and neuraminidase-treated fractions. This is probably due to altered surface charge of the enzyme molecule and easier ionic interactions with strongly polar substrate or inhibitor molecules. Somewhat higher affinity for substrate of neuraminidase-treated phosphatase may also be a result of conformational changes in the protein molecule, as indicated by diminished effective Stoke's radius. According to the "induced fit" theory of Koshland (1959), it may be possible that changes in the geometry of the desialophosphatase molecule occur more readily when substrate fits to the active site of the enzyme than in native phosphatase.

Other authors have also reported the occurrence of neuraminidase-sensitive phosphatase isoenzymes in various tissues. For instance, alkaline phosphatase from human placenta after treatment with neuraminidase loses the ability to inhibit haemagglutination of erythrocytes by H-1 virus (Ghosh, 1969). Alkaline phosphatase from sheep brain occurs in two fractions differing in NANA content (Bachhawat, 1969), affinity for some substrates, and competitive inhibition by phosphate (Saraswathi & Bachhawat, 1966).

The role of multiple forms of acid phosphatase in prostate is not known. Conceivably, the carbohydrate chain of the enzyme composed, as shown by Derechin

et al. (1971), of glucose, mannose, galactose, fucose, glucosamine and NANA, may be involved in the catalytic function of the enzyme. To what extent the pathological process in the human prostate influences heterogeneity of the enzyme molecules, and the content of sugars, remains to be elucidated.

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WŁASNOŚCI KWAŚNEJ FOSFATAZY Z PROSTATY LUDZKIEJ TRAKTOWANEJ NEURAMINIDAZĄ

Streszczenie

Wykazano, że przyczyną występowania kilku form izoenzymowych kwaśnej fosfatazy sterczu ludzkiego jest obecność kwasu *N*-acetyloneuraminowego w cząsteczce enzymu.

Potraktowanie frakcji *EI* i *EII* kwaśnej fosfatazy neuraminidazą prowadzi do powstania jednej formy molekularnej o punkcie izoelektrycznym przy pH ok. 6.0, o identycznych własnościach adsorpcyjnych oraz o promieniu Stokesa mniejszym o ok. 2.0 Å.

Wartości K_m dla *EI* i *EII* w stosunku do *p*-nitrofenylofosforanu, 5'-AMP, 2'+3'-CMP i "core" RNA drożdżowego jako substratów wykazują ten sam rząd wielkości, jakkolwiek frakcja *EII* posiada nieco wyższe powinowactwo do badanych substratów niż frakcja *EI*. Frakcje *EI* i *EII* traktowane neuraminidazą wykazują wyższe powinowactwo do substratów niż frakcje natywne.

Jony cytrynianowe wykazują wyższe działanie ochraniające przed hamowaniem fluorkiem i szybkość reakcji monochlorku jodu jest wyższa w przypadku frakcji fosfatazy traktowanych neuraminidazą.

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Addendum (added in proof). The values for NANA content presented in Table 1 are definitely lower than those which we have published earlier (Ostrowski *et al.*, 1970). As we have found recently, this was caused by contamination of the samples with slight amounts of Ampholine which increases the colour intensity with thiobarbiturate reagent in Warren reaction.

RECENZJE KSIĄŻEK

SCHERING WORKSHOP ON PHARMACOKINETICS, Berlin 1969. Advances in the Bio-
sciences, vol. 5 (G. Raspé, ed.). Pergamon Press, Oxford - Vieweg, Berlin 1970; str. 285, cena 90/- s.

Farmakokinetyka jest stosunkowo młodym, ale silnie już (zwłaszcza na zachodzie) rozwinię-
tym działem farmakologii klinicznej. Celem prowadzonych w tej dziedzinie badań jest wypracowanie
modelu tłumaczącego obserwowane zachowanie się leku i pozwalającego wytłumaczyć szereg
istotnych dla farmakologa i klinicysty problemów, takich jak szybkość i procent absorpcji leku,
czas przebywania leku w ustroju, szybkość jego wydalania i metabolizmu, oraz ilość i wielkość
dawek stosowanych przy wielokrotnym podaniu leku a koniecznych dla zapewnienia przez dłuższy
czas jego efektywnego stężenia we krwi. Kinetyczny aspekt i matematyczny opis absorpcji leków,
podziału (dystrybucji), metabolizmu i wydalania leków jest omawiany w całym szeregu obcojęzycz-
nych publikacji monograficznych i referatowych, a prace doświadczalne z tego zakresu stanowią
wiodącą problematykę badań wielu zakładów biofarmacji, farmakologii klinicznej, a także klinik
oraz laboratoriów badawczych większych firm farmaceutycznych za granicą. Miarą zainteresowania
tym zagadnieniem i przykładanego doń znaczenia może być wzrastająca stale liczba sympozjów
naukowych i konferencji stanowiących przegląd osiągnięć w tej dziedzinie.

Przedstawione w omawianej książce materiały z dwudniowej konferencji na temat farmako-
kinetyki, przeprowadzonej przez firmę Schering z udziałem takich znawców problemu jak Theorell
(Szwecja), Dost (NRF), Garrett (USA), Segre (Włochy), Dettli (Szwajcaria), Röppke i Riemann
(NRF), Bellman (USA), Gladtke (NRF), Modr i Dvoracek (Czechosłowacja), są tego najlepszym
dowodem. W szesnastu stosunkowo krótkich referatach omówiono szeroki wachlarz zagadnień,
od podstawowych definiujących zadania i metody pracy w farmakokinetyce, poprzez problemy
dotyczące czynników fizykochemicznych determinujących podział leku w ustroju i problemy
matematyczne łącznie z zastosowaniem cyfrowych i analogowych maszyn matematycznych, aż
do zagadnień szczegółowych dotyczących problemu kumulacji leku przy normalnym i uszkodzonym
systemie wydalania przez nerki, farmakokinetyki sterydów, kilku syntetycznych penicylin oraz
substancji endogennych.

Niezwykle ożywiona dyskuja, zaprotokołowana ze wszystkimi szczegółami, stanowi dodatkowy
materiał poznawczy dla zainteresowanych tą problematyką. Dająca się zaobserwować podczas niej
ostra wymiana zdań, obrona atakowanych kontrowersyjnych poglądów na kinetykę absorpcji,
podziału i eliminacji leków oraz matematycznego opisu tych zjawisk świadczy o bardzo wysokim
poziomie uczestników, którzy dali w czasie jej trwania wspaniałej klasy pokaz zaangażowania
w reprezentowaną przez siebie dziedzinę badań nad lekiem. Dowodem bogactwa treści wniesionego
przez omawianą dyskusję jest fakt, że z 284 stron wydrukowanych materiałów z tej konferencji
(włączając w tę liczbę piśmiennictwo podawane przy każdym referacie, dane biograficzne uczestni-
ków, adresy, spis treści itp.), 57 stron drukowanych petitem stanowi treść dyskusji. Podobnie ciekawa
i bogata w treść była, mająca miejsce w drugim dniu obrad, Konferencja Okrągłego Stołu.

W sumie, poświęcony farmakokinetyce V tom *Advances in the Biosciences* stanowi pozycję
godną uwagi dla każdego farmakologa, klinicysty czy farmaceuty zainteresowanego problemem
kinetyki wchłaniania, podziału i eliminacji leków. Kilka drobnych błędów matematycznych,
łatwych do zauważenia i poprawienia, w niczym nie potrafi zmniejszyć jej walorów.

Adam Danek

G.O. Aspinall, POLYSACCHARIDES. Pergamon Press, Oxford, New York, Toronto, Sidney, Braunschweig 1970; str. 228, cena $ 8.75.

Profesor G. O. Aspinall w swojej książce przedstawia zwięźle, jasno i przejrzyście aktualne wiadomości o wielocukrowcach. Treść książki podzielona jest na 14 rozdziałów, które poprzedza rozdział wstępny poświęcony nomenklaturze węglowodanów. W rozdziale pierwszym autor omawia występowanie, własności i klasyfikację polisacharydów. W drugim rozdziale opisuje metody izolowania i oczyszczania wielocukrowców oraz fizyczne i chemiczne metody stosowane w określaniu struktury tych związków. W następnych rozdziałach omówione są systematycznie poszczególne wielocukrowce: celuloza; skrobie i glikogen; glukany i fruktany; mannany, galaktomannany i glukomannany; galaktany; arabinany i ksylany; oraz glukuronany. W rozdziałach 10, 11 i 12 autor przedstawia wielocukrowce złożone.

Biosynteza prostych i rozgałęzionych wielocukrowców oraz biosynteza wielocukrowców złożonych są tematem rozdziału 13.

W opisie poszczególnych wielocukrowców autor uwzględnił budowę i własności tych związków oraz szeroko potraktował występowanie ich w przyrodzie. Liczne wykresy i schematy zamieszczone w książce bardzo ułatwiają odbiór przedstawionej treści. Każdy rozdział książki zaopatrzony jest w wykaz literatury zebranej do 1968 roku włącznie. Alfabetyczny indeks haseł zamieszczony na końcu książki ułatwia odnalezienie interesującego zagadnienia.

Ważną cechą książki jest jej zwięzłość, co pozwoliło autorowi w stosunkowo małej objętości przekazać wiele treści. Książka jest cenną pozycją jako podręczne źródło informacji o wielocukrowcach zarówno dla chemików i biochemików, jak i biologów.

Ina Gąsiorowska

PROGRESS IN BIOPHYSICS AND MOLECULAR BIOLOGY (J. A. V. Butler and D. Noble, eds.). Vol. 20. Pergamon Press, Oxford, New York, Toronto, Sidney, Braunschweig 1970; str. 341, cena $ 17.50.

Kolejny dwudziesty tom *Progress in Biophysics and Molecular Biology* zawiera pięć tematycznie odległych od siebie artykułów: 1. zagadnienie trójprzestrzennego pola elektrycznego w fizjologii komórki (R. S. Eisenberg i E. U. Johnson); 2. organizacja błon komórkowych (S. K. Malhrot); 3. replikacja wirusów zawierających DNA (A. B. Stone); 4. zastosowanie rezonansu magnetycznego w badaniu biopolimerów i ich łączenia się z jonami i małymi cząsteczkami (B. Sheard i E. M. Bradbury); oraz artykuł piąty na temat mechanizmów działania enzymów (S. Donan, C. A. Vernon i B. A. Banks).

W pierwszym artykule autorzy zajmują się zjawiskiem występowania w komórce gradientu napięcia związanego z trójwymiarowym rozprzestrzenianiem się prądu elektrycznego. W pierwszej części artykułu autorzy omawiają matematyczne ujęcie zjawisk elektrycznych zachodzących w komórce, zwracając szczególną uwagę na ich zależność od jej kształtu, a w części drugiej podają metody badania tych zjawisk w poszczególnych komórkach.

Artykuł drugi, poświęcony budowie, znaczeniu i rozmieszczeniu błon komórkowych, daje możność zapoznania się z najnowszymi odkryciami w tej dziedzinie. Autor omawia kolejno teorię struktury dwumolekularnej lipidów w błonach komórkowych, koncepcję tzw. jednostki błony komórkowej stanowiącej podstawowy składnik wszystkich błon, oraz poszczególne składniki błon.

Zadaniem trzeciego artykułu było przedstawienie nowych teorii dotyczących replikacji wirusów zawierających DNA, ze szczególnym uwzględnieniem bakteriofagów i wirusów zwierzęcych. Po stosunkowo pobieżnym opisie budowy wirusów następuje bardzo szczegółowy opis struktury DNA wirusowego, procesu replikacji, syntezy enzymów i innych białek w zainfekowanej komórce, oraz omówienie końcowego efektu działania wirusa na gospodarza, tzn. lizy komórki bakteryjnej i transformacji komórki organizmów wyższych.

Liczne dotychczas stosowane metody, takie jak badanie zjawisk osmotycznych i dyfuzji, badanie lepkości roztworów, badanie zachowania się w polu elektrycznym, pozwalają na uzyskanie wartościowych danych dotyczących właściwości cząsteczek jako całości. Żadna jednak z tych metod

nie pozwala na poznanie budowy tych cząsteczek na poziomie molekularnym. Dopiero metody spektroskopowe, a szczególnie badanie jądrowego rezonansu magnetycznego, pozwalają na badanie budowy makromolekuł, zmian ich struktury oraz nawet krótkotrwałego łączenia się ich z jonami lub związkami o niskiej masie cząsteczkowej. Zagadnieniom tym poświęcony jest artykuł czwarty. Nie jest to jednak praca dla przygodnych czytelników, ale dla badaczy zaawansowanych w tej dziedzinie, mających gruntowne przygotowanie teoretyczne i praktyczne, a pragnących zapoznać się z konkretnym zastosowaniem rezonansu magnetycznego w badaniach.

Spośród licznych ostatnio prac na temat mechanizmów działania enzymów, praca Donana i współpracowników wyróżnia się przejrzystością i zwięzłością, przy równoczesnym bardzo dokładnym omówieniu mechanizmu działania poszczególnych enzymów. Po przedstawieniu metod służących badaniu mechanizmu działania enzymów i podaniu czynników odpowiedzialnych za to działanie, autorzy przechodzą do opisu enzymów, szczególną uwagę poświęcając chymotrypsynie, karboksypeptydazie A, rybonukleazie trzustkowej oraz lizozymowi. Nie brak również interesujących wiadomości na temat izoenzymów, heteroenzymów, jak również tzw. "subform" enzymów.

Książka ta, jak zwykle już w tej serii, jest bardzo starannie wydana i będzie cennym nabytkiem w bibliotece każdego, kto zajmuje się zagadnieniami biologii molekularnej.

Marek Ombach

F. A. Steiner, NEUROTRANSMITTER UND NEUROMODULATOREN. Technik und Resultate der Mikroelektrophorese im Nervensystem. G. Thieme Verlag, Stuttgart 1971; str. 159, cena DM. 49,80.

Występowanie neurohormonów (mediatorów) w ośrodkowym układzie nerwowym i ich rola w procesach przekaźnictwa synaptycznego znajdują się w centrum uwagi nie tylko teoretyków, a więc neurofizjologów i neurofarmakologów, ale i klinicystów (psychiatrów, a w pewnej mierze również i neurologów). Mediatory pozbawione są zdolności przenikania przez barierę mózgowordzeniową, ewentualnie przenikają przez nią w ograniczonym stopniu w niektórych tylko częściach ośrodkowego układu nerwowego (np. w okolicy podwzgórza). Stąd też w badaniach nad ich wpływem na czynność neuronów stosuje się powszechnie metodę podawania mikrojonto- lub podawania elektroforetycznego.

Recenzowana monografia, należąca do zbioru monografii z zakresu psychiatrii i neurologii (Sammlung psychiatrischer und neurologischer Einzeldarstellungen; redaktorzy: W. Scheid, H. J. Weitbrecht, H. H. Wieck), opisuje metodykę i wyniki mikroelektroforetycznego podawania neurohormonów i neuromodulatorów. Publikacja składa się z trzech niejako odrębnych części. Pierwsza dotyczy metodyki mikroelektroforezy. Autor zawarł tu: 1) podstawowe zasady elektrokinetyczne leżące u podstaw mikroelektroforezy; 2) rodzaje mikroelektrod i mikropipet szklanych stosowanych w poszczególnych układach doświadczalnych, ich przygotowywanie, napełnianie i sprawdzanie; 3) roztwory stosowane do napełniania mikroelektrod; 4) prądy stosowane do elektroforezy, ich wpływ na tkankę nerwową, układy połączeń elektronicznych; 5) zasady weryfikacji histologicznej położenia mikroelektrody (miejsca odbioru); 6) zasady rejestracji (zewnątrz- i wewnątrzkomórkowej) i interpretacji uzyskanych wyników.

Część druga, zwana przez autora doświadczalną, poświęcona została omówieniu neurohormonów i neuromodulatorów. Otwierają ją rozważania nad działaniem aminokwasów na czynność komórek nerwowych. Istnieje ścisła zależność między budową chemiczną aminokwasu a jego działaniem na neuron. Przez dekarboksylację silnie pobudzających kwaśnych aminokwasów otrzymuje się aminokwasy obojętne działające silnie hamująco. Przykładami takich antagonistycznie działających par aminokwasów mogą być choćby kwas L-glutaminowy i kwas γ-aminomasłowy, jak również kwas asparaginowy i β-alanina. Autor przedstawia następnie kryteria niezbędne dla uznania substancji za neurohormon i przechodzi do szerszego omówienia wyników badań, na podstawie których przyjąć można, że związki takie jak acetylocholina, L-noradrenalina, dopamina, kwas γ-aminomasłowy, glicyna, 5-hydroksytryptamina (serotonina) oraz kwas L-glutaminowy są lub mogą być mediatorami przekaźnictwa bodźców nerwowych. W zakończeniu tej części autor omawia

modulatory, czyli związki wpływające dodatnio bądź ujemnie na pobudliwość neuronu a nie biorące udziału w samym procesie przekazywania pobudzenia lub hamowania z neuronu na neuron (powyższa definicja — podana za autorem monografii — jakkolwiek akceptowana przez wielu badaczy, może nasuwać pewne zastrzeżenia). Nie da się ich wyraźnie odróżnić od mediatorów, gdyż co najmniej niektóre z mediatorów mają funkcję modulacyjną. Ponadto za modulatory można również uważać niektóre hormony (np. sterydy nadnerczowe).

Trzecią część monografii stanowi sumaryczne omówienie uzyskanych wyników badań nad działaniem mediatorów i modulatorów na czynność neuronów poszczególnych obszarów lub jąder ośrodkowego układu nerwowego. Wyniki te zostały ponadto przedstawione schematycznie w siedemnastu tabelach zamykających publikację.

Omawiana monografia, oparta na bogatym (obejmującym z górą czterysta pozycji) piśmiennictwie, stanowi pozycję niezwykle cenną dla teoretyków pracujących nad procesami pobudzenia lub hamowania w ośrodkowym układzie nerwowym, lub też zajmujących się wpływem związków farmakologicznie czynnych na działanie poszczególnych neurohormonów lub modulatorów. Pozwala ona na szybkie zorientowanie się w aktualnym stanie i kierunkach badań (piśmiennictwo zebrane do 1969 roku włącznie), jak również ułatwia poszukiwanie materiałów źródłowych. W monografii przedstawione zostały ponadto pewne możliwości oceny wpływu niektórych związków podawanych mikroelektroforetycznie na aktywność układów enzymatycznych komórek nerwowych, co może żywo zainteresować badaczy zajmujących się biochemią tych komórek.

Wojciech Gumułka

W. L. Kretowicz, OSNOWY BIOCHIMII RASTIENIJ. Izdatielstwo ,,Wysszaja Szkoła'', wydanie V uzupełnione i poprawione; Moskwa 1971, str. 464.

Profesor W. L. Kretowicz, członek korespondent Akademii Nauk ZSRR, wicedyrektor Instytutu Biochemii im. A. N. Bacha, Akademii Nauk ZSRR w Moskwie, jest znanym biochemikiem roślin, zajmującym się od lat przemianą niskocząsteczkowych związków azotowych, a przede wszystkim przemianą aminokwasów. Obok prowadzenia podstawowych badań w zakresie biochemii prof. Kretowicz związany jest z przemysłem rolno-spożywczym, a szczególnie z przemysłem piekarniczym.

Pierwsze wydanie podręcznika *Podstawy biochemii roślin* ukazało się w r. 1952. Był to okres, kiedy w światowej literaturze podręcznikowej odczuwało się poważne braki w dziedzinie biochemii roślin. O zapotrzebowaniu na tego typu opracowanie i o jego wartości może świadczyć fakt, że podręcznik ten był tłumaczony przez wydawnictwa zagraniczne na szereg języków obcych (angielski, niemiecki, japoński, chiński, wietnamski, polski, czeski i rumuński) oraz przez wydawnictwa radzieckie na język ukraiński i gruziński. Polskie tłumaczenie I wydania ukazało się w edycji Państwowego Wydawnictwa Rolniczego i Leśnego w r. 1955.

Autor podręcznika zwrócił w swoim opracowaniu szczególną uwagę na ścisłe i różnorodne powiązania biochemii roślin z różnymi gałęziami rolnictwa, a przede wszystkim z przemysłem rolno-spożywczym.

Książka zawiera piętnaście rozdziałów, z których pierwsze 6 dotyczy ogólnych własności i budowy podstawowych składników komórki roślinnej. Rozdział 7, omawiający enzymy, jest jak gdyby pomostem łączącym pierwszą, ogólną część, z pozostałymi 8 rozdziałami omawiającymi przemiany metaboliczne komórki. Ostatni rozdział 15 obejmuje wzajemne powiązanie procesów metabolicznych organizmu oraz wpływ środowiska zewnętrznego na przemianę komórkową.

W odróżnieniu od poprzednich wydań, ostatnie uzupełniono szeregiem prostych, łatwych do zrozumienia, pomysłowych schematów, jak np. mechanizm syntezy białka, modele budowy RNA oraz niektórych białek enzymatycznych, tablica kodu genetycznego oraz schematy mechanizmów genetycznej regulacji procesów metabolicznych.

Każdy z rozdziałów kończy się spisem literatury. Pozycje literatury obejmują przede wszystkim prace przeglądowe, włącznie do r. 1970, co podnosi wartość tego podręcznika i rozszerza jego przydatność poza krąg studentów. Poza omówieniem teoretycznych podstaw określonego problemu,

wiele rozdziałów uzupełniono tabelami bądź wykresami zaczerpniętymi z oryginalnych prac badawczych, nawiązującymi do praktycznego znaczenia tych procesów dla rolnictwa czy też technologii rolno-spożywczej.

Podręcznik przeznaczony jest dla studentów uniwersytetów i wyższych szkół rolniczych o profilu technologii rolno-spożywczej. Godny podkreślenia jest duży wysiłek autora podręcznika w kierunku aktualizacji i uzupełnienia ostatniego, V wydania. Dzięki temu książka ta, której I wydanie ukazało się w r. 1952, może być nadal nowoczesną pomocą naukową dla studiujących biochemię, posiadających znajomość języka rosyjskiego.

Ponieważ książka ma charakter podręcznika przeznaczonego dla studentów, może słusznie byłoby wydzielić jeszcze jeden rozdział obejmujący zagadnienia bioenergetyki komórki, omawiający budowę, rolę i mechanizm działania związków wysokoenergetycznych oraz magazynowania i transportu energii w komórce. Problemy te są co prawda omówione w różnych rozdziałach podręcznika, wydaje się jednak, że zebranie tych danych w jeden, wydzielony rozdział ułatwiłoby studiującym zrozumienie skomplikowanego, a równocześnie bardzo istotnego, problemu gospodarki energetycznej komórki.

Kazimierz Kleczkowsk