KOMITET BIOCHEMICZNY I BIOFIZYCZNY
POLSKIEJ AKADEMII NAUK

COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS
POLISH ACADEMY OF SCIENCES

ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. 18
No. 2

WARSZAWA 1971
PAŃSTWOWE WYDAWNICTWO NAUKOWE

ADDRESS

Krakowskie Przedmieście 26/28, Warszawa 64.
Poland

Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakład 1769+131. Ark. wyd. 12,5 ark. druk. 9,125+0,125 kredy.
Papier druk. sat. kl. III, 80 g. B-1
Oddano do składania 2.II.1971. Podpisano do druku 14.VI. 1971
Druk ukończono w czerwcu 1971.
Zam. 115 U-103 Cena zł 25,—

Warszawska Drukarnia Naukowa — Warszawa, Śniadeckich 8

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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BASIC PROTEINS OF BRAIN RIBOSOMES

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The proteins extracted with 0.25 N-HCl from rabbit brain ribosomes, were separated by CM-cellulose column chromatography into five fractions which, however, were not homogeneous on starch-gel electrophoresis. The qualitative amino acid composition of the obtained fractions was similar but they differed in the lysine-to-arginine ratio, and, in contrast to total protein, they had a higher content of acidic amino acids than of the basic ones.

Basic proteins constitute a large proportion of brain tissue proteins. They form complexes with natural polyanions present in various organellae, e.g. with phospholipids in myelin sheath (Kornguth & Anderson, 1965), with DNA in nuclei (Busch, 1965), and with RNA in ribosomes (Petermann, 1964). There are numerous observations suggesting that these complexes may be involved in regulation of the synthesis of RNA (Allfrey, Littau & Mirsky, 1962), protein synthesis (Traub, Hosokawa & Nomura, 1966) and the process of myelinogenesis (Kornguth, Anderson & Scott, 1966). The strongly basic protein of myelin exhibits immunological activity and in homologous and heterologous systems evokes in experimental animals alergic encephalitis (Martenson, Deibler & Kies, 1969; Eylar & Thompson, 1969).

So far, the basic proteins of brain ribosomes are among those the least known. It seemed therefore interesting to investigate the properties of these proteins in view of the specific function and high metabolic activity of brain tissue (Krebs, 1950).

MATERIALS AND METHODS

Preparation of ribosomes. The ribosomes were obtained from brains of adult rabbits of mixed breed. The animals were killed by air embolism and then decapitated, the brain was isolated, chilled, and membranes and blood vessels were removed. All further steps of the preparation of ribosomes were carried out at 0 - 4°C. The brain was homogenized with 10 volumes of 0.44 M-sucrose - 0.004 M-$MgCl_2$ - - 0.035 M-tris buffer, pH 7.1, in a Potter-Elvehjem type homogenizer. The supernatant obtained after sedimentation of nuclei and mitochondria, was centrifuged for 40 min at 20 000 *g*. The sediment was discarded and from the supernatant, ribosomes were

obtained by two methods: (1) Precipitation at low temperature with dilute acetic acid at pH 5.1 - 5.2 (Arnstein & Cox, 1963): the sedimented ribosomes were centrifuged at 20 000 *g* and suspended in the medium *A* of Littlefield & Keller (1957) consisting of 0.25 M-sucrose, 5 mM-$MgCl_2$, 25 mM-KCl and 50 mM-tris buffer, pH 7.6. The insoluble material was centrifuged off at 12 000 *g*, and the ribosomes were again precipitated at pH 5.1. The procedure of suspending and precipitating the ribosomes was repeated 4 - 5 times (Gąsior & Szwaj, 1969). (2) Centrifugation in an ultracentrifuge (Janetzki, Vac-60, Engelsdorf, Leipzig, German Democratic Republic) at 105 000 *g* for 90 min. The sedimented ribosomes were suspended in medium *A*, the insoluble material was centrifuged off at 20 000 *g* and the ribosomes were sedimented at 130 000 *g*. Washing of the ribosomes was repeated twice.

Isolation of ribosomal protein. The ribosomes were washed several times with an ethanol-ether mixture, then with ether, and homogenized in a glass homogenizer with 0.25 N-HCl. The homogenate was left for 24 h at 4°C with constant stirring, then the extract was dialysed against water and protein was precipitated by trichloroacetic acid at 20% concentration.

Analytical methods. Total nitrogen was determined by the method of Nessler in the modification of Bürck (1960). Protein was determined spectrophotometrically (Wadell & Hill, 1956) crystalline bovine serum albumin (Koch-Light Lab., Colnbrook, Bucks., England) being used as standard. CM-cellulose column chromatography was carried out according to Johns, Phillips, Simson & Butler (1960), and starch-gel electrophoresis after Hnilica, Edwards & Hey (1966).

For determination of amino acid composition, the protein was hydrolysed with 6 N-HCl for 24 h at 110°C in sealed glass ampoules and the mixture submitted to two-dimensional paper electrochromatographic separation. The electrophoresis was carried out with the buffer of pH 2.2 according to Fishl & Segal (1963), and chromatography in a system of acetone - pyridine - *n*-butanol - diethylamine - water (15:9:15:10:16, by vol.). The basic and acidic amino acids were eluted from the electrochromatogram and determined by the ninhydrin-copper method (Fischer & Dörfel, 1953).

RESULTS

The ribosomal preparations exhibited the characteristic absorption spectrum with a maximum at 260 nm (Fig. 1), no differences being observed between the preparations obtained by several-fold precipitation at pH 5.1, and those obtained by direct high-speed centrifugation.

The amount of ribosomal protein extracted with 0.25 N-HCl from 100 g of rabbit brain, was 29 - 35 mg and the average content of nitrogen was 16.8% (Table 1). The obtained protein separated on CM-cellulose column (Fig. 2) into five fractions: fraction *X* which was not adsorbed on the column and emerged with the acetate buffer of pH 4.2; fractions f_1, f_2 and f_3 which were eluted with the stepwise increasing HCl concentration gradient; and one more fraction, designated f_4, which emerged at 0.5 N-HCl. The relative distribution of the individual fractions is presented in

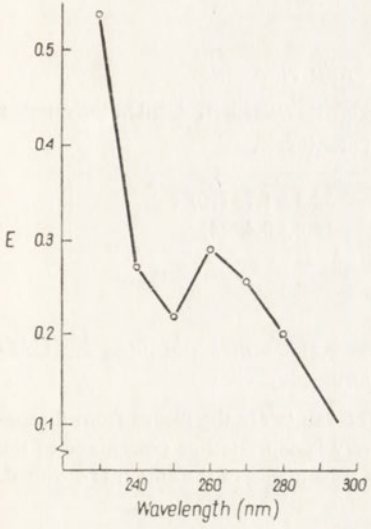

Fig. 1

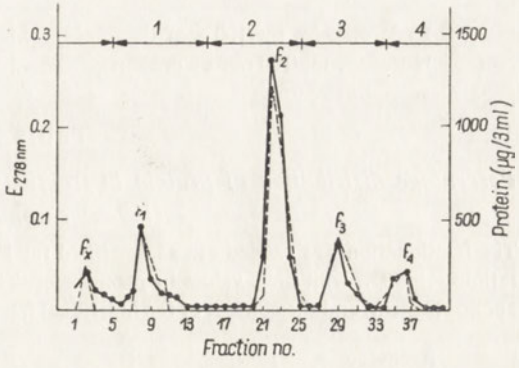

Fig. 2

Fig. 1. Absorption spectrum of the preparation of rabbit brain ribosomes obtained by the first method. In several experiments, the E_{260}/E_{280} ratio ranged from 1.35 to 1.55 and the E_{260}/E_{250} ratio from 1.22 to 1.33.

Fig. 2. CM-cellulose column chromatography of ribosomal proteins. Protein, 6 - 8 mg in acetate buffer of pH 4.2, was applied to the column (1 × 10 cm) and eluted successively with: *1*, acetate buffer of pH 4.2 containing 0.35 M-NaCl; *2*, 0.01 N-HCl; *3*, 0.02 N-HCl; *4*, 0.5 N-HCl. Fractions of 3 ml were collected, and protein was determined: ———, at 278 nm; ---, by the method of Wadell & Hill (1956).

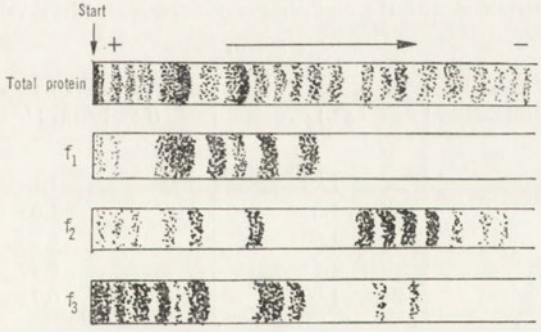

Fig. 3. Starch-gel electrophoresis of total basic proteins and the individual fractions obtained by chromatography on CM-cellulose. The electrophoresis was carried out in 0.01 N-HCl - 0.6 mM-$AlCl_3$ buffer, pH 2.2, for 15 h at 3 V/cm.

Table 2. Fraction f_2 was the quantitatively predominant one and fraction X was present in the smallest amounts. The average content of fractions f_1 and f_3 was similar. The mean recovery of the protein applied on the column amounted to about 80%.

The individual protein fractions exhibited high heterogeneity on starch-gel electrophoresis (Fig. 3). The starting protein gave more than 20 bands, and the

Table 1

Basic protein isolated from rabbit brain ribosomes

The ribosomes were obtained by methods 1 and 2. The results are mean values, ± S.D., the number of experiments being indicated in parentheses.

Basic protein, mg/100 g of tissue	32.3±1.75 (10)
Nitrogen, mg/100 mg of protein	16.8±0.45 (4)

Table 2

Percentage distribution of protein in fractions of brain ribosomes obtained by CM--cellulose chromatography

The fractionation was carried out as described for Fig. 2. The values are the means from 8 separations, ± S.D., with limit values in parentheses. The recovery is expressed as percentage of the applied protein, and the content of the individual fractions as percentages of the amount recovered.

Recovery (%)	f_x	f_1	f_2	f_3	f_4
79.5± 4.07 (72.8 - 83.6)	3.0±1.41 (0 - 5.0)	24± 3.59 (19 - 27)	37 ± 3.61 (32.3 - 43.0)	26.5± 3.85 (20.7 - 32.5)	10± 3.45 (6 - 16)

Table 3

Molar proportions of lysine to arginine and of the sum of lysine and arginine to the sum of aspartic acid and glutamic acid

After electrophoresis (see Fig. 3), the spots of the respective amino acids were eluted with methanol and their amounts determined with the ninhydrin-copper reagent. Mean values of 4 experiments are given.

Fraction	Lys : Arg	(Lys+Arg) : (Asp+Glu)
Total protein	1.24	1.12
f_1	1.37	1.03
f_2	1.66	0.86
f_3	1.11	0.89
f_4	1.00	0.75

separated fractions 9 - 13 bands each. The bands of fraction f_3 were located in the anodic part of the proteinogram and most of them had low mobility; whereas the bands of fraction f_2 had the greatest mobility toward the cathode.

The amino acid composition of the total isolated ribosomal protein and the individual fractions obtained by CM-cellulose chromatography, did not exhibit qualitative differences (Fig. 4); quantitative differences were, however, quite distinct, especially with basic and acidic amino acids. The spot of histidine, present in the total protein, was distinct only in fraction f_2. Attention should be drawn to the presence of the spot of cysteic acid (X_3). Its intensity was the greatest in fraction f_3

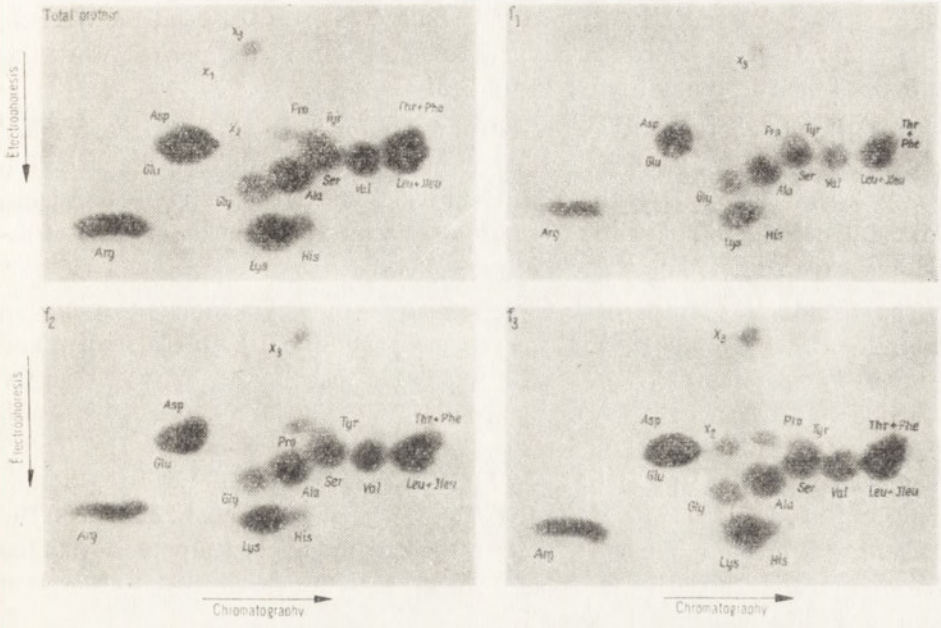

Fig. 4. Electrochromatography of the hydrolysates of basic proteins of brain ribosomes and of the individual fractions obtained by CM-cellulose chromatography. The electrophoresis was carried out in formic-acetic acid buffer, pH 2.2, for 60 min, at 18 V/cm. The chromatography was carried out in acetone - pyridine - *n*-butanol - diethylamine - water (15:9:15:10:16, by vol.).

and the smallest in f_1. The presence of cysteic acid is of interest as, apart from traces of methionine, no other sulphur amino acids were found in the protein studied.

In Table 3 are presented the molar ratios of lysine to arginine and of the sum of these two amino acids to the sum of aspartic and glutamic acids. The greatest predominance in the amount of lysine over that of arginine was found in fraction f_2, the respective ratio being 1.66, and a somewhat smaller preponderance was found in f_1, whereas f_3 and f_4 contained approximately equal amounts of these two amino acids. The ratio of the sum of lysine and arginine to the sum of aspartic and glutamic acids, in the presence of but a small amount of histidine in the protein studied, may be considered as the ratio of basic to acidic amino acids. For the total protein this ratio was higher than unity indicating its basic character, whereas for the individual fractions the obtained values were lower than unity.

DISCUSSION

The nature of the isolated ribosomal protein depends chiefly on the degree of purification of the ribosomes. One of the criteria of purity of ribosomal preparations is the absorption ratio E_{260}/E_{280} which is known to increase with purification of the ribosomes. The values for ribosomes reported by various authors were for the liver 0.7 - 1.1 (Bloemendal, Bont & Benedetti, 1964) and 1.79 - 1.92 (Gross,

Quirin-Stricker & Mandel, 1967), and for the brain 0.4 - 0.45 (Acs, Neidle & Waelsch, 1961) and 1.33 - 1.5 (Stenzel, Aronson & Rubin, 1966). In the present work this ratio was found to range from 1.35 to 1.55.

The rather low ratio of RNA to protein found for the brain may have resulted from the low content of RNA in ribosomes, which was demonstrated by Rendi & Hultin (1960). The ratio of RNA to protein in brain ribosomes is constant (Lim & Adams, 1967) although the total number of ribosomes decreases during the process of development (Adams, 1966).

The second ratio characteristic of ribosomes is the ratio of maximum to minimum absorption, i.e. E_{260}/E_{235}, and it also varies depending on the tissue studied and method of preparation. For instance, it was found to be lower for yeast than for liver ribosomes when both were prepared by the same method (Gąsior & Szwaj, 1969), whereas with different methods the ratio for liver ribosomes was 1.4 (Petermann & Pavlovec, 1963); and 1.55 - 1.79 (Gross *et al.*, 1967). In our case, the minimum appeared at 250 nm and the E_{260}/E_{250} ratio ranged from 1.22 to 1.33. The observed shift of the minimum was not caused by the applied procedures, as the liver ribosomes prepared in the same manner gave the characteristic absorption spectrum with a minimum between 235 and 240 nm.

The amount and nature of proteins isolated from ribosomes is also dependent on the method of extraction. Among others, it has been demonstrated that more protein is extracted with lithium chloride than with dilute hydrochloric acid (Neelin & Vidali, 1968), and moreover the proteins extracted at higher LiCl concentrations and in the presence of urea, are more basic (Gross *et al.*, 1967). The time of extraction is also of importance, as about 40% of the protein is extracted during the first 30 min of extraction (Wang, 1964) and it has a higher content of basic amino acids (Cohn, 1962).

The 24-h extraction with 0.25 N-HCl, applied in the present work, permitted to obtain a large amount of protein. The total unfractionated proteins possessed a slight preponderance of basic amino acids. The ratio of basic to acidic amino acids was 1.12 and the ratio of lysine to arginine, 1.24; these values are in agreement with the results of other workers for ribosomal proteins from rat liver (Butler, Cohn & Simson, 1960), pancreas (Keller, Cohen & Wade, 1964), goose erythrocyte (Neelin & Vidali, 1968), and somewhat lower than those reported for bacteria (Williams, 1967).

Heterogeneity of ribosomal proteins has been repeatedly demonstrated (Waller, 1964; Möller & Chrambach, 1967; Di Girolamo & Cammarano, 1968), different fractionation procedures being used; for instance, Amberlite CG-50 was applied for fractionation of protein from reticulocyte ribosomes (Neelin & Vidali, 1968) and CM-cellulose with *E. coli* (Williams, 1967). It seems that better results were obtained by fractionation on CM-cellulose, therefore this cationite was used in present work.

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CHARAKTERYSTYKA BIAŁEK ZASADOWYCH RYBOSOMÓW MÓZGU

Streszczenie

Białko ekstrahowane 0.25 N-HCl z rybosomów mózgu królika rozdzielono chromatograficznie na kolumnie z CM-celulozy na pięć frakcji. Przy pomocy elektroforezy w żelu skrobiowym wykazano, że frakcje te nie są homogenne. Jakościowy skład aminokwasów był wprawdzie podobny, lecz stosunek lizyny do argininy znacznie się różnił. Ponadto wykazano, że stosunek aminokwasów zasadowych do kwaśnych był niższy w poszczególnych frakcjach aniżeli w białku całkowitym.

Received 17 July, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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ISOLATION AND PROPERTIES OF ASPERGILLOPEPTIDASE FROM *ASPERGILLUS FUMIGATUS*

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1. An exocellular endopeptidase of *Aspergillus fumigatus* was isolated and a homogeneous preparation was obtained. 2. The enzyme has the following properties: molecular weight 30 700, pH optimum 7.4, temperature optimum 36°C, K_m 0.354 mM, isoelectric point 10.6. 3. The amino acid composition of the enzyme was determined; glycine and glutamic acid were found to be the *N*-terminal amino acids. 4. The enzyme specificity towards peptide bonds was studied using insulin. 5. The enzyme is inhibited by diisopropylfluorophosphate at a concentration of 10 μM, by *p*-chloromercuribenzoate and periodic acid.

A great number of moulds are known to produce appreciable amounts of various proteolytic enzymes, which have been classified as "acidic, neutral and alkaline proteases" (Bergkvist, 1963). Recent information concerning the isolation of an alkaline protease from *Aspergillus flavus* has been presented by Turková, Mikeš, Gančev & Boublik (1969) and Mikeš, Turková, Nguyen Bao-Toan & Šorm (1969).

The aim of this report is to present the isolation and some properties of the alkaline protease from *Aspergillus fumigatus*.

MATERIALS AND METHODS

Special reagents. Sephadex G-100 and G-200, particle size 40 - 120 μ (Pharmacia, Uppsala, Sweden) and CM-cellulose (Serva, Heidelberg, West Germany) were prepared according to manufacturers' instructions. Amino acids and soy bean trypsin inhibitor were from Mann Research Lab. (New York, U.S.A.). *N*-Benzoyl-DL--phenylalanine-β-naphthylamide, L-β-phenyl-α-alanyl-β-naphthylamide, benzoyl-L--tyrosinamide, *p*-tosyl-L-arginine methyl ester·HCl, γ-globulin and ovalbumin were from Koch-Light Lab. (Colnbrook, Bucks., England); *N*-α-benzoyl-DL-arginine-β--naphthylamide·HCl from Loba-Chemie (Wien, Austria); glycylleucine and tyrosylglycine from Sigma Chem. Comp. (St. Louis, Mo., U.S.A.); cytochrome *c* and α-chymotrypsin from Nutr. Biochem Corp. (Cleveland, Ohio, U.S.A.); haemoglobin

from Merck (Darmstadt, West Germany); 1,10-phenanthroline hydrate, EDTA, *p*-chloromercuribenzoate, diisopropylfluorophosphate and casein from British Drug Houses (Poole, Dorset, England). Calf thymus histone lysine-rich fraction F_1 was prepared after Johns & Butler (1962). Insulin, 5× crystallized, was from Polfa (Warszawa, Poland); other chemicals used were analytical grade products of Polish origin.

Biological material. A wild strain of *Aspergillus fumigatus* was obtained from the Department of Microbiology of the Military School of Medicine in Łódź. The *Aspergillus* was cultivated on Čapek-Dox liquid culture medium for ten days at 27°C under sterile conditions.

Acetone-dried preparation. The mycelium was separated by centrifugation at 2000 *g* and to the supernatant 1 vol. of acetone chilled to -30°C was added; then the mixture was centrifuged at 4000 *g* at 2°C, the precipitate was discarded and to the supernatant 2 vol. of cold acetone (-30°C) was added. After centrifuging at 4000 *g* at 2°C, the precipitate was collected and used for enzyme purification as described in the text.

Homogeneity of the purified aspergillopeptidase preparation was checked by (1), electrophoresis on Whatman no. 1 paper at 110 V and 4 mA in veronal - acetate buffer, pH 8.6, for 6 h; (2), starch-gel electrophoresis in borate buffer, pH 8.6, at 100 V and 57 mA for 18 h (Fowler, Colbe & Kramer, 1963); (3), polyacrylamide-gel electrophoresis in glycine buffer, pH 4.0, at 210 V and 45 mA for 8 h (McAllister, Wang & Logan, 1963; Hoagland, 1968).

Molecular weight was determined by gel filtration on Sephadex G-200 (Andrews, 1965), and by ultracentrifugation on a Spinco-Beckmann model E analytical ultracentrifuge.

Isoelectric point was determined from the electrophoretic mobility curve (Borowski & Konopa, 1957).

Amino acid analysis. The protein sample was hydrolysed at 110°C for 24, 48 and 72 h in evacuated sealed vials with distilled constant-boiling HCl, in the presence of known amounts of norleucine. Following hydrolysis, the acid was removed under reduced pressure and the residue dissolved in sodium citrate buffer of pH 2.2 (Moore & Stein, 1954; Spackman, Stein & Moore, 1958). The amino acids were determined qualitatively by electrochromatography, and quantitatively by means of a Beckmann automatic amino acid analyser (Unichrom).

N-Terminal groups were determined by the method of Sanger (Biserte, Holleman & San tiere, 1960).

Enzyme assays. Proteolytic activity measurements were made by the method of Anson (1938) using denatured haemoglobin in phosphate buffer, pH 7.6, as substrate. The pH and temperature optima were determined at the initial reaction rate, The enzyme activity is expressed in Anson units per 1 mg of protein.

The specificity of the enzyme was examined by the fingerprinting method. Crystalline bovine insulin, 25 mg, and 0.1 mg of the enzyme were incubated in 0.2 M-phosphate buffer, pH 7.4, at 36°C for 6 h, and then freeze-dried in solid CO_2. The formed oligopeptides were separated by paper electrochromatography. The electro-

phoresis was carried out on Whatman no. 3 *MM* paper in a system of pyridine - - acetic acid - water (25:25:950, by vol.), pH 4.75, at 2000 V and 14 mA for 3 h at 16°C. The chromatography was carried out in *n*-butanol - pyridine - acetic acid - - water (15:10:3:12, by vol.) for 22 h at 16°C. The amino acid composition of the oligopeptides was determined, after hydrolysis, by high-voltage electrochromatography at 2000 V and 60 mA according to Clegg & Naughton (1965).

In the experiments on the effect of metal ions and some other compounds, the enzyme solution was preincubated for 15 min with the compound studied, then proteolytic activity measurements were made during 30 min at 5 min intervals by the method of Kunitz (1947).

Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), from the nitrogen content, and by measuring the extinction at E_{280}.

RESULTS

Enzyme purification. The acetone-dried preparation of *Aspergillus fumigatus* culture medium was extracted with 0.9% NaCl solution, dialysed for 48 h against water at 2°C, and the dialysis residue (activity 13 Hb units/mg protein) resolved into 11 protein fractions by Sephadex G-200 gel filtration (Fig. 1). The activity was found in two peaks; fraction *D* was the richest in protein but had only a slight activity (14 units/mg protein) whereas practically the whole activity corresponded to fraction *F* (220 units). Fraction *F* was collected, freeze-dried, dissolved in 5 ml of cold 0.15 M-KCl solution, and submitted to second gel filtration on Sephadex G-100 (Fig. 2). Of the 7 protein fractions obtained, only one peak, *E'*, exhibited the activity (228 units). On CM-cellulose chromatography this fraction was resolved into 4 peaks (Fig. 3); practically all activity was recovered in fraction *D''* (221 units), and only traces of activity were found in fraction *C''* (5.3 units).

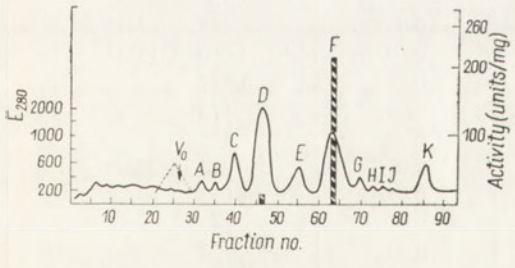

Fig. 1

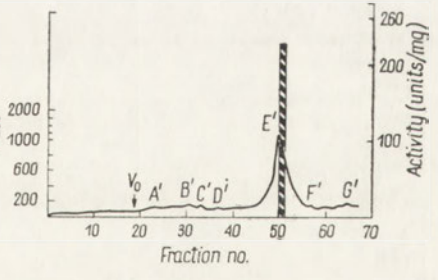

Fig. 2

Fig. 1. Gel filtration on Sephadex G-200 of aspergillopeptidase Af. The enzyme preparation, 140 mg of protein, was applied to the column (50×2.5 cm) which had been equilibrated with 0.054 M-tris--KCl buffer, pH 7.4. The elution was performed with 0.15 M-tris-KCl buffer, pH 7.4. The flow rate was 80 ml/h and fractions of 3 ml were collected.

Fig. 2. Gel filtration on Sephadex G-100 of fraction *F* (see Fig. 1). The sample, 125 mg of protein, was applied to the column (50×2.5 cm) which was equilibrated and eluted as described for Fig. 1.

The purified enzyme fraction *D''* was freeze-dried and used for further experiments.

Physical properties of aspergillopeptidase Af. The purified enzyme preparation was homogeneous on paper, starch-gel and polyacrylamide-gel electrophoresis (Figs. 4, 5, 6). The sedimentation pattern in the ultracentrifuge demonstrated the

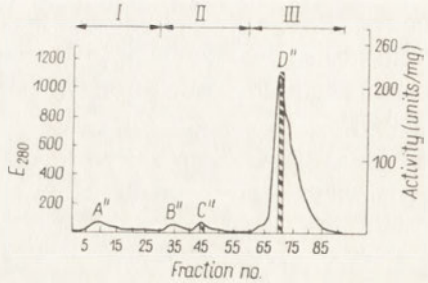

Fig. 3. CM-cellulose chromatography of fraction *E'* (see Fig. 2). The sample, 100 mg of protein, was eluted with: *I*, 150 ml of 0.05 M-Na-acetate - phosphate buffer, pH 4.85; *II*, 150 ml of 0.3 M--Na-acetate - phosphate buffer, pH 5.0; *III*, 150 ml of 0.3 M-Na-acetate-phosphate buffer, pH 6.0. The flow rate was 36 ml/h and fractions of 3 ml were collected.

Table 1

Amino acid composition of the aspergillopeptidase Af

The analyses of amino acid composition were carried out on the Beckmann automatic amino acid analyser.

Amino acid	Residues		Molecular weight of amino acids
	per molecule	in μmoles	
Lysine	139	1.39	20 294
Histidine	1	0.01	155
Arginine	1	0.01	174
Aspartic acid	13	0.13	1 729
Threonine	5	0.05	595 (625)*
Serine	10	0.10	1 050 (1155)*
Glutamic acid	11	0.11	1 617
Proline	8	0.08	920
Alanine	9	0.09	801
Cystine (half)	2	0.02	240 (252)*
Valine	9	0.09	1 053
Methionine	1	0.01	149
Isoleucine	4	0.04	524
Leucine	4	0.04	524
Tyrosine	2	0.02	362 (380)*
Phenylalanine	2	0.02	330
Glycine	16	0.16	1 200
NH_3	150	1.50	2 250
Total	237		33 967 (34 114)*

* Correction applied for decomposition of amino acids on hydrolysis.

Table 2

Activity of aspergillopeptidase Af toward different synthetic substrates

Substrate	Result of reaction
L-β-Phenyl-α-alanyl-β-naphthylamide	positive
Benzoyl-L-tyrosinamide	positive
N-Benzoyl-DL-phenyl-α-alanine-β-naphthylamide	negative
p-Tosyl-L-arginine methyl ester·HCl	negative
N-α-Benzoyl-DL-arginine-β-naphthylamide·HCl	negative
Glycylleucine	positive
Tyrosylglycine	positive
Casein (control)	positive
Insulin (control)	positive

Table 3

The effect of different compounds on the proteolytic activity of the purified aspergillopeptidase Af

The enzyme was preincubated with the compounds tested and after 15 min the activity was determined.

Addition	Concentration (mM)	Enzyme activity (%)	Inhibition (%)
None, control (non-dialysed enzyme)	—	100	0
1,10-Phenanthroline*	1.0	100	0
EDTA*	0.08	100	0
$CoCl_2$	0.1	75	25
$MnSO_4$	0.1	80	20
$MgSO_4$	0.1	100	0
$CuSO_4$	0.1	95	5
H_5IO_6	1.0	60	40
p-Chloromercuribenzoate	1.0	0	100
$CaCl_2$	0.1	100	0
$MgCl_2$	1.0	5	95
$FeCl_3$	1.0	84	16
$ICH_2 \cdot COOH$	10.0	65	35
$NiSO_4$	0.1	90	10
$ZnSO_4$	1.0	85	15
Diisopropylfluorophosphate	0.01	0	100
Soy bean trypsin inhibitor	0.02%	100	0
Trasylol	5000 i.u.	100	0
ε-Amino caproic acid	100.0	100	0

* Enzyme dialysed for 48 h.

homogeneity of the obtained enzyme preparation (Fig. 7). The sedimentation coefficient was $S = 2.96 \times 10^{-13}$ sec, and the diffusion coefficient $D = 7.2 \times 10^{-7}$ cm^2 sec^{-1}. The molecular weight obtained from the centrifugation data was 30 700, minimum molecular weight calculated from the amino acid composition 34 114, and obtained by gel filtration on Sephadex G-200, 28 000 (Fig. 8). In the last case, the difference of 2000 in comparison with the two former methods is within the limits of error of the gel-filtration method. The isoelectric point of the enzyme was 10.6 (Fig. 9).

Amino acid analysis showed that glycine and glutamic acid were the *N*-terminal amino acids. The results obtained with the acid hydrolysate of the enzyme preparation on the automatic amino acid analyser are shown in Table 1; by two-dimensional paper chromatography the same amino acids were detected, except arginine and isoleucine which migrated on paper together with histidine and leucine, respectively (Fig. 10). The enzyme preparation was characterized by a very high content of lysine residues, 58% of all amino acid residues. Such a high content of lysine in the enzyme molecule enables the appearance of polylysine fragments with the free amino groups with p*K* value of 10.63 ($-NH_3$) in its structure.

Enzyme activity toward haemoglobin had a temperature optimum at 36°C and pH optimum at 7.4. It should be noted that although the isoelectric point of the enzyme was as high as 10.8, the optimum pH value was 7.4. The shift towards acidity of the pH optimum in relation to p*I* suggests the dependence of the activity of the *Asp. fumigatus* enzyme upon the decreasing number of dissociated amino groups.

The isolated proteinase was thermolabile and lost entirely its activity on incubation at 45°C for 30 min.

The Michaelis constant calculated from the Lineweaver-Burk plot (Fig. 11) was 0.354 mM.

The specificity of aspergillopeptidase Af was tested on five synthetic substrates and two peptides, Gly-Leu and Tyr-Gly, insulin and casein being used in control experiments. The results presented in Table 2 show that of the synthetic substrates tested only L-*β*-phenyl-*α*-alanyl-*β*-naphthylamide and benzoyltyrosinamide were decomposed. Gly-Leu and Tyr-Gly were slightly decomposed.

Fig. 4. Paper electrophoresis of: *A*, enzyme preparation (0.28 mg of protein), and *B*, human blood serum (1.4 mg). Conditions: Veronal - acetate buffer, pH 8.6, 6 h, 110 V, 4 mA. *S*, start.

Fig. 5. Starch-gel electrophoresis of *A*, enzyme preparation (0.28 mg of protein) and *B*, blood serum (1.4 mg). Conditions: borate buffer, pH 8.6, 18 h, 100 V, 57 mA.

Fig. 6. Polyacrylamide-gel electrophoresis of *A*, enzyme preparation (10 µg of protein) and *B*, fraction F_1 of calf thymus histone (50 µg of protein). The electrophoresis was carried out in glycine buffer, pH 4.0, at 210 V and 45 mA for 8 h. *1*, Boundary of the gel column; *2*, band of the enzyme studied.

Fig. 7. Ultracentrifugation sedimentation patterns of the enzyme preparation (0.1% solution in phosphate buffer, pH 7.6) at 23°C. The photographs were taken when the rotor speed reached 59 780 r.p.m. (64 min) and then at 10 min intervals.

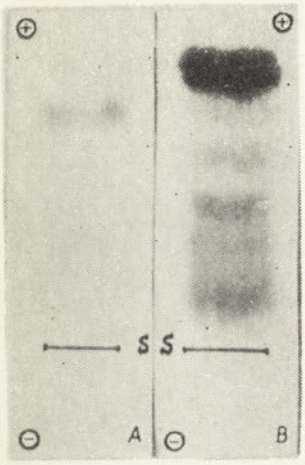

Fig. 4

Fig. 5

Fig. 6

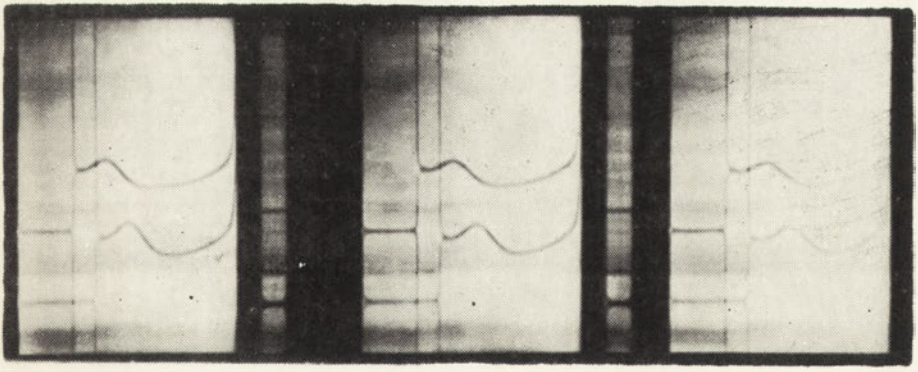

Fig. 7

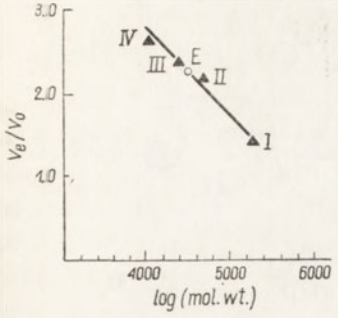

Fig. 8

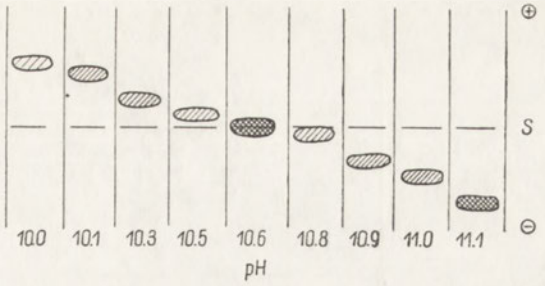

Fig. 9

Fig. 8. Determination of molecular weight of aspergillopeptidase Af by Sephadex G-200 gel filtration. The standards: *I*, γ-globulin; *II*, ovalbumin; *III*, α-chymotrypsin; and *IV*, cytochrome *c*, were applied at concentrations of 1 mg/ml. *E*, enzyme.

Fig. 9. Determination of isoelectric point of aspergillopeptidase Af. Electrophoretic mobility was determined in carbonate - bicarbonate buffer, μ 0.1, at 2°C.

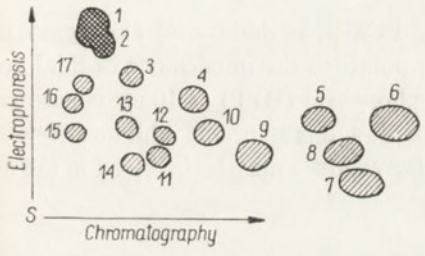

Fig. 10

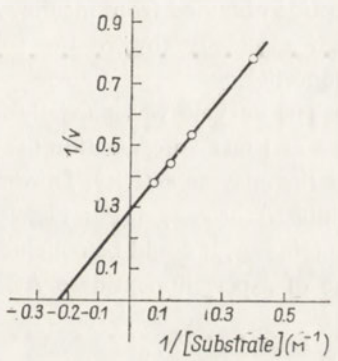

Fig. 11

Fig. 10. Electrochromatogram of the acid hydrolysate of aspergillopeptidase Af. The electrophoresis was carried out in acetate buffer (CH_3COOH - HCOOH - water; 200:60:740, by vol.), pH 1.5, at 1000 V and 21 mA for 4 h at 16°C. The chromatography was carried out in *n*-butan-1-ol - - CH_3COOH -water (4:1:1, by vol.) for 22 h at 16°C. *1*, Lys; *2*, His; *3*, Gly; *4*, Ala; *5*, Val; *6*, Leu (Ile); *7*, Phe; *8*, Met; *9*, Tyr; *10*, Pro; *11*, Glu; *12*, Thr; *13*, Ser; *14*, Asp; *15*, Cys; *16*, and *17*, non--identified.

Fig. 11. Graphic determination of Michaelis constant by the Lineweaver-Burk plot. The enzyme concentration was 0.05 mg protein per sample, haemoglobin concentration ranged from 0.20 mM to 0.80 mM. The activity is expressed as μmoles of the decomposed substrate per minute.

The products of insulin breakdown were analysed by the fingerprinting method (Fig. 12). It was found that 10 oligopeptides were formed; the amino acid composition of one of them (*VI*) is presented in Fig. 13. The obtained results indicate that the enzyme shows specificity toward peptide bonds formed between the following amino acids: Leu-Cys, Val-Glu, Val-Lys, Thr-Pro, Leu-Glu, Cys-Ser, Cys-Ala (Fig. 14). This was confirmed by the determination of *N*-terminal amino acids of the oligo-

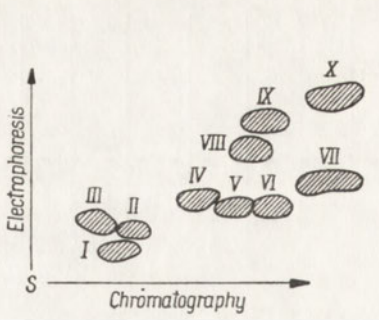

Fig. 12

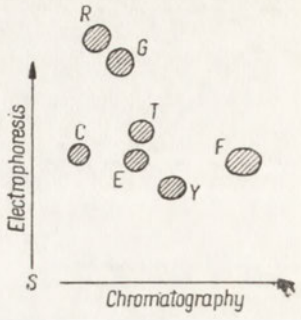

Fig. 13

Fig. 12. Electrochromatographic analysis ("fingerprinting") of the products obtained after digestion of insulin by aspergillopeptidase Af. The electrophoresis and chromatography were carried out as described in Methods. The numbers denote the individual peptides (see Fig. 14).

Fig. 13. Electrochromatographic separation of oligopeptide *VI* (see Fig. 12) obtained after digestion of insulin by aspergillopeptidase Af. Conditions of electrophoresis and chromatography as described for Fig. 10.

peptide obtained from insulin. As can be concluded, the enzyme does not show too high specificity toward the substrate and is characterized by the endopeptidase properties.

The enzyme was partially inhibited by PCMB, periodic acid and heavy metal ions at 1 mM concentration (Table 3). This points to the importance of thiol groups for the enzyme activity. Diisopropylfluorophosphate (DFP) at 10 μM concentration inhibited the enzyme completely. As this compound is a specific inhibitor of "serine" proteinases, it could be concluded that serine residues may be involved in the active site of aspergillopeptidase Af.

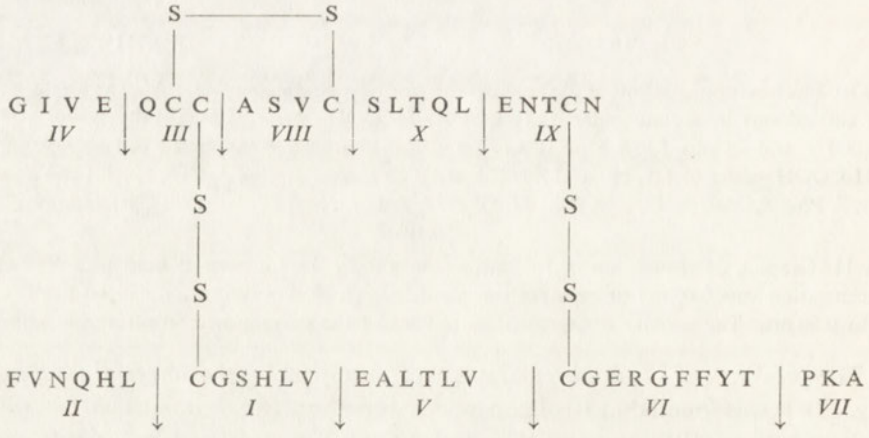

Fig. 14. The insulin model, represented in amino acid symbols according to IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry*, 7, 2703, 1968). The arrows indicate the bonds broken by aspergillopeptidase Af, and the numbers correspond to the oligopeptides shown in Fig. 12.

DISCUSSION

It seems advantageous to make a detailed comparison between the physico-chemical and enzymic properties of the proteinase isolated from *Aspergillus fumigatus*, and those of other proteolytic enzymes obtained from various *Aspergillus* species by different authors. So far, five proteinases have been isolated from this genus. Their amino acid composition and molecular weight, in comparison with those of our preparation, are presented in Table 4. The molecular weight of aspergillopeptidase Af corresponds to that obtained by Ichishima & Yoshida (1965) for aspergillopeptidase A from *Aspergillus Saitoi*. It also contains the same number of cysteine residues per molecule.

Table 4

Comparison of aspergillopeptidase Af with other proteinases from genus Aspergilli

Amino acid	Alkaline proteinase *Asp. flavus* Turková *et al.* (1969)	Alkaline proteinase *Asp. sojae* Hayashi *et al.* (1967)	Aspergillopeptidase A *Asp. Saitoi* Ichishima & Yoshida (1965, 1966)	Aspergillopeptidase B *Asp. oryzae* Subramanian & Kalnitsky (1964)	Aspergillopeptidase C *Asp. oryzae* Nordwig & Jahn (1966, 1968)	Aspergillopeptidase Af *Asp. fumigatus* Present work
N-terminal	Gly		Ser	Gly		Glu Gly
C-terminal	Ala		Ala	Ala		
Lysine	11	14	11	11 - 12	12	139
Histidine	3 - 4	5	3	4	4	1
Arginine	2	3	1	2	3	1
Aspartic acid	21	31	34 - 35	21	21 - 22	13
Threonine	11	18	25	11	13	5
Serine	20	28	42 - 43	19	23	10
Glutamic acid	12 - 13	19	22	12	13	11
Proline	4 - 5	6	10	4	5 - 6	8
Glycine	20	27	31 - 32	19	21	16
Alanine	23	32	20	23	23	9
Cysteine	0	2	2	0	0	2
Valine	15	18	22	15	16	9
Methionine	1	2	0	0	1	1
Isoleucine	9 - 10	14	11 - 12	9 - 10	10 - 11	4
Leucine	9	14	19 - 20	9	10	4
Tyrosine	5	8	17 - 18	5	4 - 5	2
Phenylalanine	5	7	13	5	6	2
Tryptophan	2	2	1	2	2	0
Total	177	250	283 - 289	171 - 173	187 - 191	237
Mol. wt.	18 000	25 750	34 800	17 000	19 650	30 700

It should be noted that glycine was found to be the *N*-terminal amino acid in the alkaline proteinase from *Aspergillus flavus* (Turková *et al.*, 1969) and in aspergillopeptidase B from *Aspergillus oryzae* (Subramanian & Kalnitsky, 1964), whereas in our aspergillopeptidase Af both glycine and glutamic acid were found to be *N*-terminal. This may suggest the presence of two polypeptide chains in the enzyme molecule.

Mikeš *et al.* (1969) established the following amino acid sequence in the active site of alkaline proteinases: Gly-Thr-Ser-Met-Ala. The same amino acid sequence in the active site is characteristic of proteinases from French beans (Shaw & Wells, 1967) and brewer's yeast (Shaw & Ruscica, 1968). The amino acid sequences of active sites of the known "serine" proteinases of microorganisms are: for *Sorangium sp.* protease Asp-Ser-Gly (Whitaker, Jurášek & Roy, 1966); and for *Streptomyces griseus* pronase Asp-Ser-Gly (Whälby, 1968). In the light of these data it would appear that the presence of serine residue in the active site of aspergillopeptidase Af is not an exceptional phenomenon.

The author wishes to express his gratitude to Prof. Dr. M. Kański for his continuous help and valuable advice given in the course of this work; to Prof. Dr. K. Zakrzewski of the Warsaw Serum and Vaccine Plant for making available the ultracentrifuge; and to Prof. Dr. A. Adamiec of the Institute of Pharmacy, Warszawa, for enabling the analysis of the amino acid composition on the automatic analyser.

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IZOLOWANIE I WŁAŚCIWOŚCI ASPERGILLOPEPTYDAZY Af Z *ASPERGILLUS FUMIGATUS*

Streszczenie

1. Wyizolowano zewnątrzkomórkową endopeptydazę z płynnego podłoża *Aspergillus fumigatus*.

2. Enzym posiada następujące właściwości: optimum pH 7,4, optimum temperatury 36°C, K_m $3{,}54 \times 10^{-4}$ M, ciężar cząsteczkowy 30 700, punkt izoelektryczny 10.6.

3. Oznaczono skład aminokwasowy enzymu i stwierdzono, że aminokwasami *N*-końcowymi są glicyna i kwas glutaminowy.

4. Specyficzność enzymu wobec wiązań peptydowych badano przy użyciu insuliny.

5. Inhibitorami enzymu są diizopropylofluorofosforan, *p*-chlorobenzoesan rtęci oraz kwas nadjodowy.

Received 5 September, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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THE INFLUENCE OF BASIC PROTEINS ON POLYSOMES. STUDIES ON THE REACTIVATION OF POLYSOMES INHIBITED BY HISTONES IN A CELL-FREE SYSTEM

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Inhibition of polysomes caused by histones in a cell-free protein biosynthetic system can be reversed (in 80%) by short treatment with trypsin. Poly U or exogenous mRNA also reverse such inhibition but ribosomal RNA with no template activity does not possess this property. It is suggested that inactivation of polysomes by histones results in inhibition of polysomal mRNA without a loss of ribosome activity or degradation of mRNA molecules. The action of trypsin and exogenous mRNA in restoration of the activity of histone-blocked polysomes is discussed.

During the past few years valuable information has been gathered on the role of basic proteins in the regulation of protein biosynthesis in cell nuclei. Allfrey & Mirsky (1958), Allfrey, Littau & Mirsky (1963) and Bonner, Huang & Gilden (1963) elucidated to a great extent the role of histones in the process of transcription occurring in the cell nuclei. The strong inhibitory effect of histones on amino acid incorporation into nuclear ribosomal protein has also been demonstrated. Inhibition by histones of protein biosynthesis outside the nucleus, i.e. in the cytoplasm, was also observed by Berlinguet & Normand (1968), Kruh & Labie (1968) and Toczko, Sieliwanowicz & Jachymczyk (1969), but the mechanism of this inhibition has not yet been explained.

On the other hand, Monroy, Maggio & Rinaldi (1965), Östner & Hultin (1968), Salb & Marcus (1963) and Mano & Nagano (1966) have demonstrated that protein biosynthesis in cell-free systems isolated from sea urchin embryos, rat liver and metaphasal HeLa cells is activated by treatment of ribosomal fraction with low trypsin concentrations. The results of these studies have shown that in the cell cytoplasm of different organisms there exists a protein synthesizing system which is inactive as a result of inhibition with basic proteins.

The purpose of the present study was to investigate whether the blocking of the protein biosynthesis process by histones *in vitro* at the polysomal level can be reverted by the action of trypsin or exogenous mRNA. It may be expected that the results obtained would serve as a basis for an attempt at explaining the mechanism of the polysome inactivation by histones.

MATERIALS AND METHODS

Poly U, ATP, GTP, creatine phosphokinase, trypsin (2×cryst.) and ribonuclease A from bovine pancreas were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); creatine phosphate from Calbiochem (Los Angeles, California, U.S.A.); Difco protein hydrolysate (vitamin-free casamino acids) from Difco Laboratories Inc. (Detroit, Mich., U.S.A.); reduced glutathione from Dr. Theodor Schuchardt (München, West Germany). L-[U-^{14}C]phenylalanine (spec. act. 468 mCi/mmol) and mixture of ^{14}C-labelled amino acids (spec. act. 52 mCi/milliatom of carbon) from the Radiochemical Centre (Amersham, England). Messenger RNA (mRNA) and ribosomal RNA (rRNA) were prepared from the cotyledons of peanut seeds (*Arachis hypogea* L. var. Virginia 56-R) germinated for 2 - 4 days according to Jachymczyk & Cherry (1968), lyophilized and stored in sealed tubes at -20°. All reagents were of analytical grade.

The histones used for the inhibition of protein biosynthesis *in vitro* were obtained from hog renal cortex nucleohistones by Toczko (1969). They showed remarkable similarity in their amino acid composition, electrophoretic properties and *N*-terminal amino acids to the homologous histones from calf thymus studied by Johns (1964) and Hnilica (1966). On polyacrylamide-gel electrophoresis at pH 2.4 the investigated histones showed the presence of only five typical fractions namely $f_3, f_1, f_{2b}, f_{2a2}$ and f_{2a1}. The lack of any other bands on the electrophoretograms shows that the histones used in the present studies were free of contamination by other proteins.

Preparation of ribosomes and supernatant. Rats of both sexes of the Wistar strain were used, generally 3 months old (200 - 300 g). The animals were fasted overnight, then killed by decapitation. The liver was quickly excised and dropped into an ice-cold solution of medium *A* (0.25 M-sucrose, 0.005 M-$MgCl_2$, 0.025 M-KCl, 0.05 M-tris-HCl, pH 7.8). After cooling, the liver was minced and homogenized in 2.5 vol. of medium *A* in a glass Potter-Elvehjem type homogenizer with a teflon pestle. The homogenate was centrifuged at 15 000 *g* for 10 min. The supernatant fraction was filtered through glass wool, mixed with 10% sodium deoxycholate solution to a final concentration of 1% deoxycholate, and the polyribosomal fraction was prepared according to the method of Wettstein, Staehelin & Noll (1963). After centrifugation at 105 000 *g* for 3 h in a VAC-60 ultracentrifuge (H. Janetzki, Leipzig, German Democratic Republic), the pellets from a few tubes were pooled, suspended in medium *B* (0.25 M-sucrose, 0.005 M-$MgCl_2$, 0.025 M-tris-HCl, pH 7.8 and 0.01 M-$KHCO_3$), centrifuged at 15 000 *g* for 5 min for clarification and diluted in medium *B* to obtain the final concentration of ribosomes of 10 mg/ml. Ribosomal protein and RNA concentrations were determined by the method of Tester & Dure (1966). By this technique E_{260} value of 12 is equivalent to 1 mg of ribosomes and 0.5 mg of RNA.

To obtain cell sap free of ribosomes, the 15 000 *g* supernatant of the homogenate (without deoxycholate) was centrifuged at 105 000 *g* for 90 min and the upper half of the supernatant was used immediately for experiments. All operations were carried out at 0 - 4°.

Amino acid incorporation in vitro. The incubation medium contained the following components (in μmoles/ml of medium): tris-HCl buffer, pH 7.8, 50; KCl, 5; $KHCO_3$, 10; $MgCl_2$, 7; glutathione, reduced, 10; ATP, 1; GTP, 0.25; creatine phosphate, 10; and creatine phosphate kinase, 50 μg. The medium contained either 0.5 μCi of uniformly labelled L-[^{14}C]phenylalanine or 0.5 μCi of the mixture of uniformly ^{14}C-labelled amino acids. The concentration of ribosomes, supernatant proteins, histones, trypsin and different kinds of RNA used as template RNA varied; detailed information is given in the legends to Figures and Tables. The amino acid incorporation systems were incubated at 37° for 45 min.

Assay for incorporated label. Amino acid incorporation was stopped by placing a 0.1 ml portion of the incubation mixture on Whatman 3*MM* filter paper disc which was then dried in a stream of hot air for 20 sec. Discs were placed in cold 10% trichloroacetic acid solution containing approx. 2% of Difco protein hydrolysate or 0.1 M-phenylalanine, and left for at least 3 h prior to further treatment according to the method of Mans & Novelli (1961). Radioactivity was assayed by means of a Liquid Scintillation Spectrometer Tri-Carb, Packard, Model 1003. Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

The study of histones for ribonucleolytic activity. The determination of the ribonucleolytic activity in the histones isolated from hog renal cortex was carried out according to the method of Anfinsen, Redfield, Choate, Page & Caroll (1954) adapted for the determination of low enzyme activity by Kruh & Labie (1968).

RESULTS AND DISCUSSION

The ribonucleolytic activity of the histone preparation. This was studied by the method of Anfinsen, adapted by Kruh & Labie (1968) for the determination of low enzyme activity; it was found that the activity of 0.01 μg of ribonuclease, i.e. the lowest activity detectable by means of this method, corresponds to about 1.6 mg of histones. From the studies of Kruh & Labie it follows that the polysomes undergo detectable destruction at ribonuclease concentrations exceeding 0.02 μg per 1.6 mg of ribosomes. In the present studies not more than 300 μg of histones (i.e. 0.002 μg of ribonuclease), were used per 1 mg of ribosomes; this addition of ribonuclease could have no effect on the inhibition of protein biosynthesis.

The binding of histones by rat liver ribosomes. In order to study the quantity of histones that can be bound to the ribosomal fraction isolated from rat liver, an experiment was performed in which increasing amounts of histones were added to 1 mg of ribosomes and after centrifugation of the obtained mixtures, the quantity of protein in the supernatant was determined. The results of the experiment shown in Table 1 indicate that the addition of 0.2 mg to 0.85 mg of histones to 1 mg of the ribosomal fraction did not cause an increase of the protein content in the supernatant, which shows that 1 mg of ribosomes can bind completely as much as 0.85 mg of histones. With a further increase in the amount of histones added (from 0.85 to

Table 1

Binding of histones by ribosomes

Ribosomes (1 mg) were dissolved in 1 ml of medium *B* and mixed with various quantities of histone solution as indicated in the Table. The reaction mixtures were made up to 2 ml with medium *B* and centrifuged for 1 h at 165 000 *g*. Protein in the supernatant was determined according to the method of Lowry *et al.* (1951) with the standard curve made for histones. The quantity of free histones in the supernatant was calculated by subtracting the quantity of protein in the supernatant after centrifugation of 1 mg of intact ribosomes (145 μg of protein) in 2 ml of medium *B* from the total amount of supernatant protein.

Histones added (μg)	Histones in the supernatant (μg)
200	—
300	—
500	—
750	—
850	—
950	3
1000	45
1100	98

1.1 mg/mg of ribosomes), they could not be bound completely and began to appear in the supernatant.

Effect of histones on protein biosynthesis in vitro. As shown in Fig. 1, the incorporation of radioactive L-amino acids into protein was inversely proportional to the concentration of histones. The addition of 300 μg of histones into the cell -free system which contained 1 mg of ribosomes caused 76% inhibition. This amount of histones, which corresponded to 1/3 of the amount of histones that could be bound completely by 1 mg of ribosomes, was used throughout all the experiments.

Effect of trypsin on the reactivation of histone-bound ribosomes. According to the results of the experiments of Östner & Hultin (1968), rat liver ribosomes treated with trypsin (concentration ranging from 0.05 μg to 0.2 μg per mg of ribosomes) for 10 min at 35° show a significant increase of activity in amino acid incorporation into protein. In these studies the greatest increase of activity was observed at a trypsin concentration of 0.1 μg per mg of ribosomes. A greater amount of trypsin caused a rapid decrease of ribosome activity.

When studying the influence of trypsin on the activity of the ribosomes which had been inhibited with histones (300 μg/mg of ribosomes) a similar phenomenon was observed, with the exception that the amount of trypsin needed to obtain similar activity was significantly higher and was about 5 μg of the enzyme per 1 mg of inactivated ribosomes. This trypsin concentration was used in the experiments presented in Table 2. In these experiments two control systems were used: 1) with intact rat liver ribosomes, 2) with ribosomes activated with trypsin. The proper samples contained: 3) ribosomes treated with histones at 0°, 4) ribosomes incubated during 40 min at 37° after having been treated with histones and 5) ribosomes incubated as in 4 and treated with trypsin.

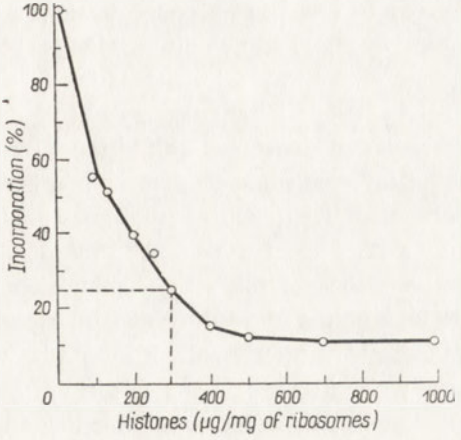

Fig. 1. Inhibition by histones of incorporation of L-[^{14}C]amino acids into protein *in vitro*. The incorporation system contained in 1 ml: 1 mg of ribosomes mixed with various quantities of histones, 0.5 μCi of the mixture of [^{14}C]amino acids (spec. act. 52 mCi/mA) and 0.75 mg of the 105 000 *g* supernatant protein. Other additions as described in Materials and Methods. The incubation was carried out for 45 min at 37°.

Table 2

Effect of trypsin on the activation of rat liver ribosomes, intact and inhibited by histones

Ribosomes were mixed with histones dissolved previously in medium *B* (300 μg of histones per 1 mg of ribosomes), diluted with the same medium to a final volume of 1 ml and kept in an ice bath for further incorporation assay or preincubated for 40 min at 37°. After incubation, the samples were divided in two parts. One part was used immediately for the amino acid incorporation studies; to the other part 0.1 ml of trypsin (5 μg) was added, followed by the addition of 0.9 ml of medium *B* and the reaction mixture was incubated for 20 min at 37°. For control experiment, 1 mg of ribosomes (without histones) was incubated with 0.1 μg of trypsin for 20 min at 37°. Ribosomes were isolated from incubation medium by centrifugation at 165 000 *g* for 45 min. The ribosomal pellets were rinsed twice with 0.3 ml of medium *B* and suspended in 0.2 ml of the same medium. The incorporation system contained in 1 ml: 0.8 mg of the 105 000 *g* supernatant protein, 0.6 mg of ribosomes and 0.5 μCi of the mixture of ^{14}C-labelled amino acids (52 mCi/mA). Other additions were as described in Materials and Methods.

Expt. no.	Pretreatment of ribosomes	Trypsin (μg)	Incorporation (c.p.m./mg ribosomes)	
			expt. I	expt. II
1	None (control)	—	3924	3420
2	None (control)	0.1	5321	4430
3	300 μg histones; 40 min, 0°	—	548	370
4	300 μg histones; 40 min at 37°	—	720	570
5	300 μg histones; 40 min at 37°	5.0	4010	3650

The ribosomes subjected to low trypsin concentrations increased their activity in amino acid incorporation by about 30%. This activity has been taken into account when calculating the degree of the reversal of inhibition of ribosomes treated with histones. The addition of histones to the ribosomes at 0° decreased their activity in about 85%. After incubation of ribosomes with histones for 40 min at 37°, a slight increase of activity of the investigated system in comparison with the analogous system kept at 0° was observed. Treatment of ribosomes previously incubated with histones for 40 min at 37° with 5 μg of trypsin per 1 mg of blocked ribosomes restored their activity to about 80% of the activity of the control system activated by trypsin. This shows that in the studied system no ribonuclease activity could have been present. On the basis of these results it should be accepted that the inhibition by histones did not cause irreversible changes in the system and that histones do not cause the destruction of polysomes by specific degradation of mRNA as it has been suggested by Kruh & Labie (1968). Furthermore, the removal of histones by trypsin resulted in an almost complete reactivation. The inhibition of protein biosynthesis by histones *in vitro* has therefore to be related to attachment of these proteins to polysomes.

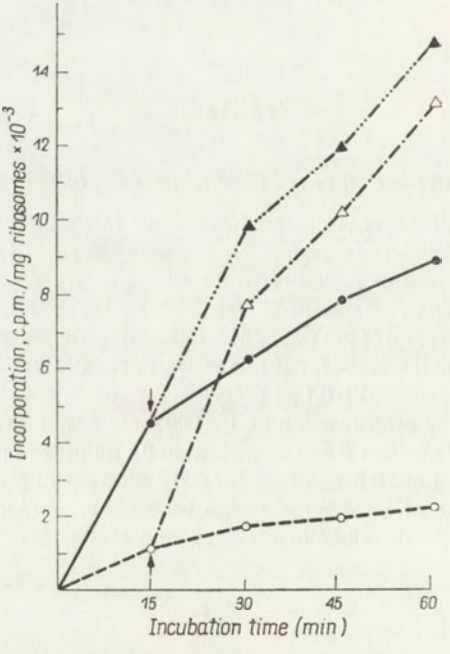

Fig. 2. Effect of poly U on the L-[^{14}C]phenylalanine incorporation into protein in the presence of histones. Ribosomes (0.5 mg) were treated with 150 μg of histones at 0°C before using them in the incorporation assay. The incubation medium contained in 1 ml: 0.5 mg of intact ribosomes (control) or 0.5 mg of ribosomes inhibited by histones, 0.7 mg of the 105 000 *g* supernatant protein and 0.5 μCi of L-[^{14}C]phenylalanine (468 mCi/mmol). Poly U (100 μg) was added 15 min after the beginning of the incubation. ●, Intact ribosomes; ○, ribosomes treated with histones; ▲, intact ribosomes with poly U; △, ribosomes treated with histones, and poly U. Arrows indicate the addition of poly U.

Effect of mRNA on the activation of a system containing ribosomes blocked by histones. The ribosomal fraction inactivated by histones could be activated not only by trypsin treatment but also by an addition of new mRNA (Fig. 2). The addition of poly U stimulated very effectively the incorporation of radioactive phenylalanine in the system with ribosomes blocked by histones and the incorporation was even higher than in the system containing no histones.

A similar effect was observed after the addition of exogenous mRNA obtained from germinated peanut cotyledons (Table 3a). The addition of mRNA to the control system resulted in only a relatively small increase of activity, whereas the addition of the same amount of mRNA to the system containing ribosomes blocked by histones caused an increase of activity, exceeding (similarly as in the case of poly U) the incorporation in the control. The replacement of mRNA by an equal amount of ribosomal RNA possessing no template activity (Table 3b) had no effect on the blocked system.

In view of these results it seems that the inactivation of the investigated system can occur by the blocking of polysomal mRNA by histones without any apparent change in activity of the ribosomes as such, since the addition of poly U or mRNA causes a significant increase in their ability to incorporate amino acids. As the addition of ribosomal RNA had no effect, it may be concluded that activation by exogenous RNA was not due to its ability to detach the histones from the blocked system, but rather to formation of new polysomes. Our results do not contradict the results obtained by Berlinguet & Normand (1968) who demonstrated a possibility of de-

Table 3

Effects of mRNA and rRNA on the incorporation of [^{14}C]*amino acids in the system with intact ribosomes and ribosomes inhibited by histones*

Messenger RNA (mRNA) and ribosomal RNA (rRNA) were prepared from peanut cotyledons according to Jachymczyk & Cherry (1968). Ribosomes were treated with histones (300 µg per 1 mg of ribosomes) as described in the legend of Table 1. The incubation medium contained in 1 ml: 1 mg of intact (control) or histone-treated ribosomes, 0.8 mg of 105 000 *g* supernatant protein, 50 µg mRNA or rRNA. Other additions were as described in Materials and Methods. Incubation was conducted for 45 min at 37°.

Pretreatment of ribosomes	Addition of		Incorporation (c.p.m./mg ribosomes)		
	mRNA (µg)	rRNA (µg)	expt. I	expt. II	expt. III
a None (control)	—	—	2085	2433	2129
None (control)	50	—	3450	3675	2640
+300 µg histones	—	—	296	420	391
+300 µg histones	50	—	2350	2670	2287
b None (control)	—	—	1097	2183	
None (control)	—	50	1204	2200	
+300 µg histones	—	—	231	265	
+300 µg histones	—	50	264	275	

tachment of histones by heparin. Heparin, owing to the presence of $-SO_3H^-$ groups, has a stronger polyanionic character than mRNA.

Reactivation by trypsin of the system blocked by histones as well as the activation by the addition of exogenous mRNA with high template activity seems to indicate that the blocking of polysomes by histones could be connected with the partial liberation of ribosomes from polysomes. Introduction of a new active mRNA causes the reassociation of ribosomes into polysomes; such new active mRNA can be exogenous, or endogenous, i.e. freed from histones after trypsin treatment.

The authors are grateful to Professor Dr. Irena Chmielewska for encouragement, stimulating discussions and for comments on the manuscript, and to Dr. K. Toczko for the gift of highly purified histones from hog renal cortex.

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WPŁYW BIAŁEK ZASADOWYCH NA POLISOMY. BADANIA NAD REAKTYWACJĄ POLISOMÓW ZINAKTYWOWANYCH PRZEZ HISTONY

Streszczenie

Wykazano, że hamowanie przez histony procesu biosyntezy białka w układzie bezkomórkowym może być zniesione (w 80%) przez krótkotrwałe potraktowanie trypsyną zinaktywowanych układów polisomalnych. Poli U lub mRNA powoduje przywrócenie aktywności zablokowanego układu, natomiast rybosomalny RNA nie wywołuje żadnych zmian aktywności. Sugeruje się, że inaktywujące działanie histonów polega na blokowaniu polisomalnego mRNA bez jego degradacji i przy zachowaniu niezmienionej aktywności rybosomów. Przedyskutowano mechanizm reaktywowania układu zablokowanego histonami pod wpływem trypsyny i egzogennego mRNA.

Received 2 November, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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ACTIVATION OF RIBOSOMES FROM PEA SEEDS BY TRYPSIN

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Trypsin treatment of the ribosomal fractions from the cotyledons of dry pea seeds results in an eightfold increase in activity. Similar fractions from the cotyledons of germinated pea seeds show also an increase in activity after trypsin treatment but this increase is lower and inversely dependent on the level of the initial activity of the studied ribosomal fractions. The occurrence of mRNA in association with a protein inhibitor of translation in ribosomal fractions isolated from pea seeds is discussed.

There is evidence that, in animal cells, molecules of mRNA can exist not as naked strands of RNA but in association with protein, that is as ribonucleoprotein particles which may be inactive in protein biosynthesis (Spirin, 1966, 1969). Occurrence of similar forms of mRNA in plants has not been yet fully proved. Dure & Waters (1965) have shown that seeds can germinate in the presence of actinomycin D. This antibiotic while inhibiting RNA synthesis in imbibing seeds, including mRNA synthesis, exerts no influence on the rate of protein synthesis in them. Marcus & Feely (1964, 1965) and Jachymczyk & Cherry (1968) have shown that the lack of an active form of mRNA is the only factor limiting the initiation of protein biosynthesis in dry seeds. So far there is no direct proof which could militate for one of the two possible mechanisms: synthesis of the new mRNA or activation of the mRNA still existing in dry seeds.

The activation in cell-free system of inactive ribosomal preparations from unfertilized sea urchin eggs (Monroy, Maggio & Rinaldi, 1965), HeLa cells at the stage of metaphase (Salb & Marcus, 1965) and rat liver (Östner & Hultin, 1968) by trypsin treatment, enabled to demonstrate the existence of the inactive complexes of mRNA--protein.

It seemed, therefore, of interest to investigate whether the ribosomal fraction isolated from cotyledons of pea seeds could be activated by trypsin.

MATERIALS AND METHODS

Preparation of ribosomes and supernatant. Pea seeds were germinated on moist paper in constant humidity at 25°C in a dark chamber. Cotyledons were excised from pea seedlings which had germinated 10, 24, 60 and 120 h, or from dry seeds. Cotyledons were ground in a precooled at -10°C mortar with medium A (0.5 M-sucrose, 0.01 M-$MgCl_2$, 0.02 M-KCl, 0.05 M-tris-HCl buffer, pH 7.8, 0.005 M-2-mercaptoethanol), 3 ml of the medium being used per 1 g of cotyledons. The homogenate was strained through cheesecloth and centrifuged for 15 min at 20 000 *g*.

To obtain ribosomes, to the 20 000 *g* supernatant fraction 10% deoxycholate solution was added to a final concentration of 1%; 5.5 ml of the solution was layered over 4.5 ml of 2 M-sucrose containing 0.01 M-$MgCl_2$, 0.02 M-KCl, 0.005 M-2-mercaptoethanol and 0.01 M-tris-HCl buffer, pH 7.8, and centrifuged at 105 000 *g* for 3 h in a VAC-60 ultracentrifuge (H. Janetzki, Leipzig, German Democratic Republic). The pellets pooled from a few tubes were resuspended in medium B (0.01 M-$MgCl_2$, 0.02 M-KCl, 0.005 M-2-mercaptoethanol and 0.01 M-tris-HCl buffer, pH 7.8), centrifuged for 10 min at 20 000 *g* and the clear supernatant diluted to a final concentration of ribosomes containing 2 - 4 mg of RNA/ml.

Ribosomal protein and RNA concentrations were determined by the method of Tester & Dure (1966). By this technique E_{260} value of 12 is equivalent to 1 mg of ribosomes and 0.5 mg of RNA.

The 105 000 *g* supernatant (enzymic fraction) was prepared from maize shoots germinated in the dark for 2 - 4 days. The shoots were homogenized with medium A as above, centrifuged for 15 min at 20 000 *g* and the supernatant (without the addition of deoxycholate) was centrifuged again at 105 000 *g* for 90 min. The upper one-third of the supernatant was collected and either used immediately or frozen in small portions in solid CO_2 - acetone and stored at -20°C. All operations were made at 0 - 4°C.

Incubation of ribosomes with trypsin. Samples containing 1 - 2 mg of ribosomes were incubated for 20 min at 37°C with serial dilutions of trypsin (Sigma Chem. Co., St. Louis, Mo., U.S.A.) (0.01 - 2 µg/mg ribosomes in 1 ml). The incubation was stopped by chilling the reaction mixture to 0°C, which was followed by the addition of ice-cold medium B to a final volume of 10 ml, and the obtained solution was centrifuged immediately for 60 min at 165 000 *g*. The ribosomal pellets were washed twice with 0.3 ml of medium B, suspended in 0.2 ml of the same medium and used for further studies.

Amino acid incorporation in vitro. The conditions for the incorporation of ^{14}C-labelled amino acids were essentially those used by Jachymczyk & Cherry (1968). The reaction was stopped by placing a 100 µl portion of the incubation medium on a Whatman 3*MM* filter paper disc, drying in a stream of hot air for 20 sec and submerging it into a cold 10% trichloroacetic acid solution containing approx. 2% Difco protein hydrolysate (vitamin-free casamino-acids, Difco Lab. Inc. Detroit, Mich., U.S.A.), and left for at least 3 h prior to further treatment.

The amount of radioactive amino acids incorporated into protein was deter-

mined by the method of Mans & Novelli (1961). Radioactivity was assayed by means of a Packard liquid scintillation spectrometer, model 1003.

Total protein was determined by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Activity of the ribosomal fraction isolated from cotyledons of dry pea seeds in incorporation of ^{14}C-labelled amino acids into protein in the cell-free system amounted only to about 200 c.p.m./0.1 mg of ribosomal protein. This fraction, when subjected to trypsin treatment, showed a considerable increase of the activity at lower enzyme concentrations, up to 1 μg of trypsin per 1 mg of ribosomes; however, the activity decreased with a further increase in trypsin concentration (Fig. 1a).

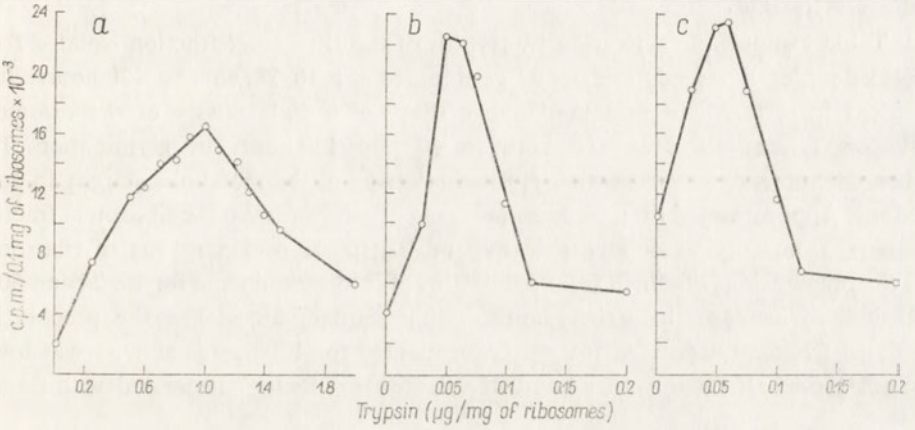

Fig. 1. Effect of trypsin on the activity of the ribosomal fraction from cotyledons of dry pea seeds (*a*), of seeds germinated during 10 h (*b*) and 60 h (*c*). The medium for amino acid incorporation contained the 105 000 *g* supernatant from maize shoots (0.3 mg of protein), 0.4 mg of ribosomes and 0.5 mCi of the mixture of L-[U-^{14}C]amino acids (Radiochemical Centre, Amersham, England).

Thus it seems that the proteins which blocked the activity of the ribosomal fraction were either more susceptible to trypsin attack, or more accessible to the enzyme than other ribosomal proteins. It should be also taken into account that trypsin often contained traces of ribonuclease which could inhibit the ribosomal activity when the higher concentrations of trypsin were used.

In ribosomes from cotyledons of germinating seeds, the initial activity increased with the time of germination. After 10 and 60 h of germination, the activity was, respectively, 400 and 1150 c.p.m./0.1 mg of ribosomes, which is twice and six times as much as in ribosomes from dry seeds (Table 1). Treatment with trypsin increased severalfold the activity, to the greatest extent for seeds germinated for 10 h. The highest activity, independent of the time of germination, was obtained at a trypsin concentration of 0.06 μg/mg of ribosomal protein (Fig. 1b,c). With a further increase

in trypsin concentration the activity decreased and at 1.2 μg of trypsin for seeds germinated for 10 h it fell to the control value, and for seeds germinated for 60 h, it was even lower (Fig. 1b,c).

It should be noted that in ribosomes from cotyledons of dry seeds the highest activity was observed at the trypsin concentration of 1 μg per 1 mg of ribosomes, whereas in the case of germinating seeds the activity was the highest in the presence of 0.06 μg of trypsin. This observation suggests that the quantity of trypsin required for activation of ribosomal fraction was dependent, in a given preparation, on the overall content of proteins, both those occurring in ribosomes *in vivo* and those isolated together with ribosomes. Besides starch, protein is a main reserve depot in cotyledons of dry seeds of Papilionaceae plants, thus isolation of ribosomes may result in extraction of a considerable admixture of these proteins. This may cause a necessity of application of greater amounts of the enzyme in the process of ribosomes activation.

Table 1 shows the activation by trypsin of the ribosomal fraction isolated from cotyledons of dry seeds and seeds germinated for 10, 24, 60 and 120 hours. It is evident from the data presented above that the initial activity of the examined ribosomal fractions decreased between 60 and 120 hours of germination. This phenomenon is connected with a gradual lowering of the metabolic activity in cotyledons. Application of 1.0 μg trypsin per 1 mg of ribosomes to the ribosomal fraction isolated from cotyledons of dry seeds, and 0.05 μg trypsin per 1 mg of ribosomes to the preparations obtained from cotyledons of seeds germinated for 10, 24 and 60 h, resulted in increases in activity approaching similar values. For the preparation isolated from cotyledons of pea seeds germinated for 120 h, this activity was lower by at least a half. On the other hand, the "activation factor" decreased with time of

Table 1

Effect of trypsin on the activation of ribosomal fractions from the cotyledons of dry and germinated pea seeds

Ribosomes from dry seeds were treated with 1 μg of trypsin per 1 mg of ribosomes and those from germinated seeds with 0.05 μg of trypsin per 1 mg of ribosomes. The incorporation system contained in 1 ml: 0.3 mg of 105 000 *g* supernatant protein from maize shoots and 0.3 mg of ribosomes. Other additions as described in Materials and Methods. The figures are mean values from three different experiments; the dispersion fell within 2 - 6%.

Time of germination (hours)	Activity (c.p.m./0.1 mg of ribosomes)		Activation factor
	control	after trypsin treatment	
0	205	1640	8
10	390	2200	6
24	600	2145	4
60	1125	2245	2
120	428	880	2

germination, ranging from 8 for the ribosomal fraction isolated from dry seeds, to 2 for the fraction isolated after 60 h of germination. Thus, an inverse relationship between the activity of the ribosomal preparations and the degree of their activation by trypsin could be observed at the first stage of germination. The activation factor had the same value in the case of trypsin activation of ribosomal fractions isolated from five-day-old pea shoots as well as from rat liver (results not shown).

The results presented above, together with the earlier observations of Marcus & Feely (1964, 1965) and Jachymczyk & Cherry (1968) suggest that the main factor limiting the activity of the protein biosynthetic system in cotyledons of dry seeds is not a lack of mRNA but rather its existence in association with protein molecules and therefore unavailable for translation. Thus, the activating effect of trypsin would consist in a release of mRNA from inactive ribonucleoprotein complexes. The amount of the inhibitor diminished with the time of germination which was reflected by an increase in the initial activity and in a decrease in the degree of activation by trypsin. This fact implies that a certain portion of blocked mRNA can occur in cells of germinated seeds, similar to that found in rat liver cells (Henshaw, 1968). It may be suggested that the ratio of initial activity of the ribosomal fraction to that after treatment with trypsin may characterize the degree of utilization of the genetic information present in cytoplasmic mRNA.

The authors are deeply grateful to Professor Irena Chmielewska for her encouragement and continued interest in this work and for helpful criticism of the manuscript. Thanks are also due to Professor Jan Kielanowski, director of the Institute of Animal Physiology and Nutrition of the Polish Academy of Sciences, for making available the use of the ultracentrifuge in his Institute.

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AKTYWOWANIE RYBOSOMÓW NASION GROCHU POD WPŁYWEM TRYPSYNY

Streszczenie

Wykazano, że frakcja rybosomalna z liścieni suchych nasion grochu pod wpływem trypsyny zwiększa ośmiokrotnie aktywność we włączaniu radioaktywnych aminokwasów do białek. Analogiczne preparaty rybosomalne wydzielone z liścieni kiełkowanych nasion grochu również zwiększają aktywność pod wpływem trypsyny, lecz osiągany wzrost aktywności jest niższy i w sposób odwrotnie proporcjonalny zależny od aktywności początkowej badanych frakcji. Przedyskutowano możliwość występowania we frakcji rybosomalnej nasion grochu połączeń mRNA z białkowym inhibitorem translacji.

Received 22 October, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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ADENOSINE-5′-MONOPHOSPHATE AMINOHYDROLASE OF HUMAN PLACENTA

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Deamination and dephosphorylation of 5′-AMP, as well as deamination of adenosine by human placental cytoplasm were studied. It was found that the cytoplasm liberated more ammonia from 5′-AMP than from adenosine. The activities toward the two substrates were partly separated by dialysis against water. The formation of 5′-IMP as a product of deamination of 5′-AMP was demonstrated by paper chromatography. The results obtained indicate that human placenta (virtually free of blood), in addition to adenosine aminohydrolase, contains specific AMP-aminohydrolase (EC 3.5.4.6).

Hayashi, Baldridge, Olmsted & Kimmel (1964) were unable to demonstrate deamination of 5′-AMP to 5′-IMP in human placenta, and they concluded that this tissue does not contain specific 5′-AMP aminohydrolase (Hayashi & Olmsted, 1965).

As AMP-aminohydrolase (EC 3.5.4.6) is a very widespread enzyme in animal tissues, an attempt has been made to reinvestigate its presence in human placenta. The present paper describes the results of this study.

MATERIALS AND METHODS

Preparation of placental cytoplasm. Fresh human placenta was freed of membranes and scraped with scissors. The scraped tissue was washed three times with 2 liters of physiological saline and twice with 300 ml portions of 0.3 M-sucrose, blotted on filter paper and disintegrated in a meat grinder, weighed and homogenized by hand with 2 volumes of 0.3 M-sucrose in a glass-teflon Potter-type homogenizer. For short homogenization 10 strokes of a rather loose pestle were applied; long homogenization was performed by additional 20 strokes of a more tight pestle. The homogenate was filtered through a double layer of gauze and centrifuged for 10 min at 8000 *g* at 4°C. The supernatant was centrifuged again for 30 min at 105 000 *g* at 4°C in Janetzki VAC-50 ultracentrifuge (Engelsdorf, Leipzig, German Democratic Republic). The supernatant fraction called cytoplasm was used for experiments.

The blood clot taken from surface of the placenta, was treated in the same way. All the preparative steps were performed in a cold room at 4°C.

Enzyme assays. The formation of ammonia from 5′-AMP and adenosine as well as liberation of inorganic phosphate from 5′-AMP were studied in the same medium at a temperature of 30°C. The standard incubation mixture contained in a final volume of 2 ml: 100 μmoles of potassium-succinate buffer of pH 6.5, 200 μmoles of KCl, 10 μmoles of 5′-AMP potassium salt or 10 μmoles of adenosine. The reaction was started by addition of cytoplasm and stopped with 1 ml of 15% (w/v) trichloroacetic acid. After 10 min the sample was adjusted to pH about 5 by addition of 0.5 ml of 1.8 N-KOH and diluted with ammonia-free water to a final volume of 5 or 10 ml. The precipitated protein was removed by centrifugation for 10 min at 1000 *g* and in the clear supernatant fluid, ammonia and inorganic phosphate were determined. All assays were performed in duplicate for three time periods (15, 30, 45 min). Under conditions employed the linear relationship between the amount of product formed and the time of incubation was observed. The results are expressed in nmoles of NH_3 or P_i formed/min/mg of protein.

Analytical methods. Ammonia was determined by the phenol-hypochlorite method according to Chaney & Marbach (1962). The colour was developed for 30 min at room temperature.

Inorganic phosphate was determined according to Gomori (1941-1942).

Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine albumin, fraction V as standard.

Haemoglobin was determined as cyanmethaemoglobin with Drabkin's reagent according to Brus, Pawelski & Murawski (1967). The commercial standard solution of cyanmethaemoglobin was used for calibration curve. The extinction was read at 540 nm in Unicam SP-500 spectrophotometer in cuvettes of 4 cm light-path.

Chromatographic separation of adenosine, inosine, 5′-AMP and 5′-IMP was carried out by descending paper chromatography on Whatman no. 1 paper in saturated ammonium sulphate - isopropanol - water (79:2:19, by vol.) according to Deutsch & Nilsson (1953). The spots were visualized by photography in UV light.

Reagents. All nucleotides, nucleosides and bovine serum albumin (fraction V) were supplied by Sigma Chem. Comp. (St. Louis, Mo., U.S.A.). Standard solution of cyanmethaemoglobin was supplied by Biomed (Kraków, Poland). Amberlite IR-120 was a B.D.H. (Poole, Dorset, England) product. Other reagents were supplied by Polskie Odczynniki Chemiczne (Gliwice, Poland).

In all experiments, ammonia-free water was used; it was obtained by passing glass-distilled water through a column (2.5×45 cm) of Amberlite IR-120.

RESULTS

Placental cytoplasm, when incubated with 5′-AMP or adenosine, produced ammonia. With 5′-AMP as substrate, inorganic phosphate was also liberated. As it may be seen from the data presented in Table 1, longer homogenization resulted

Table 1

Formation of ammonia and inorganic phosphate from 5′-AMP and adenosine by placental cytoplasm

The incubation mixture consisted of: 50 mM-potassium succinate buffer, pH 6.5, 100 mM-KCl, 5 mM-5′-AMP potassium salt, or 5 mM-adenosine, or both; final volume 2 ml. The reaction was started by addition of 0.5 ml of cytoplasm. For details see text. The results represent mean values, ±S.D., from the number of experiments indicated in brackets. Statistical differences were measured by Student's *t* test.

Homogenization	Cytoplasm protein (mg/ml)	Substrate			Ratio (NH_3 formed from 5′-AMP): :(NH_3 formed from adenosine)	Substrate	
		5′-AMP	Adenosine	5′-AMP+ adenosine		5′-AMP	5′-AMP+ adenosine
		NH_3 (nmol/min/mg protein)				P_i (nmol/min/mg protein)	
Short	3.8±0.6 (7)	9.4±3.8 (7)	2.4±0.9 (7)	9.0±4.9 (5)	4.2±1.1 (7)	15.7±4.3 (7)	13.6±2.2 (4)
Long	6.9±0.4 (6)	5.7±1.3 (6)	2.8±1.1 (6)	—	2.3±1.0 (6)	16.8±4.9 (6)	—
P	<0.05	<0.05	>0.4		<0.01	>0.7	

in about twice as high protein concentration in the cytoplasm, and about twice as low amount of ammonia produced from 5′-AMP per 1 min and per 1 mg of protein, than did shorter homogenization. The activities per 1 mg of protein of adenosine aminohydrolase and 5′-nucleotidase were the same in cytoplasms obtained both after short and long homogenization. In every experiment 5′-AMP was a better source of ammonia than adenosine. The mean ratio of nmoles of ammonia per 1 min and per 1 mg of protein produced from 5′-AMP to that produced from adenosine in cytoplasms after short and long homogenization was 4.2 and 2.3, respectively. This indicates that AMP-aminohydrolase was extracted more readily than adenosine aminohydrolase or 5′-nucleotidase.

When 5′-AMP and adenosine were added together to the cytoplasm, the amount of ammonia formed was the same as with 5′-AMP alone ($P > 0.3$), but exceeded about 4 times the amount of ammonia produced when adenosine was the sole substrate ($P < 0.001$).

The data presented in Table 1 seem to indicate that deamination of 5′-AMP proceeded not only after its dephosphorylation to adenosine but also directly through the reaction catalysed by AMP-aminohydrolase.

As it is known that erythrocytes contain AMP-aminohydrolase (Rao, Hara & Askari, 1968), a careful control was performed to exclude the possibility that the low activity of AMP-aminohydrolase observed was due to contamination by blood of the placental cytoplasm. When placental tissue had not been washed at all, the

cytoplasm obtained contained more protein and consequently liberated less ammonia per 1 mg of protein from 5′-AMP and adenosine, as compared with the cytoplasm obtained from the tissue washed in the usual manner (Table 2). The cytoplasm obtained from the blood clot produced less ammonia per 1 mg of protein from 5′-AMP and adenosine than the cytoplasm from washed placental tissue.

The haemoglobin content was estimated in the cytoplasm both from the blood clot and the washed placental tissue after short and long homogenization (Table 3). The amount of haemoglobin in placental cytoplasm was negligible, being lower in the cytoplasm obtained after short homogenization, i.e. in the cytoplasm which liberated the greatest amount of ammonia from 5′-AMP per 1 mg of protein. The

Table 2

Liberation of ammonia and inorganic phosphate from 5′-AMP and adenosine by washed and non-washed placental tissue and blood clot

One placenta was used for each experiment, short homogenization being employed. Experimental conditions as described in Table 1 and in the text.

Material	Exp. no.	Cytoplasm protein (mg/ml)	Substrate				
			5′-AMP	Adenosine	5′-AMP+ adenosine	5′-AMP	5′-AMP+ adenosine
			NH_3 (nmol/min/mg protein)			P_i (nmol/min/mg protein)	
Non-washed placental tissue	1	10.8	5.8	1.2	5.6	6.6	6.2
	2	7.9	3.1	0.7	—	6.8	6.8
Washed placental tissue	1	4.5	9.2	3.3	9.2	13.1	12.2
	2	2.8	5.5	1.2	5.5	17.8	15.7
Blood clot	1	19.2	5.0	1.0	5.7	3.0	2.0
	2	14.6	3.4	0.9	3.4	0.4	0.4

Table 3

Haemoglobin content and ammonia formation from 5′-AMP and adenosine by cytoplasm obtained from blood clot and placental tissue

The experiment was performed on a single placenta. Placental tissue was washed and homogenized as described in Methods. For experimental conditions see Table 1 and the text.

Material	Cytoplasm protein (mg/ml)	Cytoplasm haemoglobin (mg/100 ml of extract)	Substrate	
			5′-AMP	Adenosine
			NH_3 (nmol/min/mg protein)	
Blood clot	22.2	1200	4.5	0.6
Placental cytoplasm				
Short homogenization	3.5	28	9.9	2.0
Long homogenization	7.4	58	5.9	2.0

higher haemoglobin content in the cytoplasm after longer homogenization seems to have been caused by the extraction of myoglobin as a result of extensive homogenization.

The results presented in Tables 2 and 3 show that there was but negligible contamination by blood of the placental cytoplasm obtained from washed placenta.

As AMP-aminohydrolase from other tissues is strongly inhibited by fluoride (Makarewicz, 1969) whereas adenosine aminohydrolase is affected to a much lesser extent (Pfrogner, 1967), the inhibition by fluoride of ammonia formation from 5′-AMP and adenosine was studied (Table 4). The effect of EDTA, which is known to inhibit 5′-AMP dephosphorylation (Nechiporenko, Goncharenko & Goloborodko, 1966), was also investigated. In the presence of 20 mM-fluoride, the activity of AMP-aminohydrolase was inhibited, and about the same amounts of ammonia were formed from 5′-AMP and adenosine, whereas in the absence of fluoride the ratio of ammonia formed from 5′-AMP to that from adenosine was about 4. EDTA in 5 mM concentration inhibited dephosphorylation of 5′-AMP in about 50%, but had only a slight inhibitory effect on deamination of 5′-AMP or adenosine.

Table 4

Effect of fluoride and EDTA on deamination of adenosine and 5′-AMP and dephosphorylation of 5′-AMP by placental cytoplasm

Experimental conditions as described in Table 1.

Addition	Substrate			
	5′-AMP	Adenosine	5′-AMP + adenosine	5′-AMP
	NH_3 (nmol/min/mg protein)			P_i (nmol/min/mg protein)
None (control)	5.1	1.3	5.2	15.3
20 mM-NaF	1.1	0.8	1.9	11.5
None (control)	6.6	1.9	—	13.2
5 mM-EDTA	5.7	1.7	—	6.1

Table 5

The aminohydrolase and nucleosidase activities in placental cytoplasmic fractions

The conditions of separation by dialysis as described in the text.

Material	Protein (mg/ml)	Substrate		
		5′-AMP	Adenosine	5′-AMP
		NH_3 (nmol/min/mg protein)		P_i (nmol/min/mg protein)
Undialysed cytoplasm	6.6	4.2	1.9	25.8
Globulin fraction	6.7	30.0	0.7	27.2
Albumin fraction	3.6	5.2	2.6	27.8

In a number of tissues the separation of adenosine aminohydrolase and AMP--aminohydrolase could be achieved by simple dialysis against water (Żydowo, 1960). Such an experiment was performed with placental cytoplasm obtained after long homogenization, containing 6.6 mg of protein per 1 ml. A 20-ml sample of cytoplasm was dialysed against three changes of water, 5 liters each, at 4°C for 24 h. Then the precipitate formed was separated by centrifugation. The supernatant fraction was collected, and the precipitate dissolved in a small volume of water containing KCl. In the two fractions obtained, called the "albumin fraction" and "globulin fraction", the enzymic activities were estimated (Table 5).

Complete separation of the two aminohydrolase activities has not been achieved but the globulin fraction liberated about 40 times more ammonia from 5′-AMP than from adenosine, whereas the activity of adenosine aminohydrolase was lowest in the globulin fraction and highest in the albumin one. The activity of 5′-nucleotidase per 1 mg of protein was the same in both fractions.

To check the enzymically formed products, the two protein fractions were incubated with 5′-AMP and adenosine and, after stopping the reaction by placing the tubes in a boiling water bath for 5 min, the nucleotides and nucleosides present in the incubation mixture were separated by paper chromatography. It may be seen from Fig. 1 that the globulin fraction converted 5′-AMP to adenosine, 5′-IMP and inosine. The presence of the spot of 5′-IMP on the chromatogram is a proof that deamination of 5′-AMP to 5′-IMP by the action of AMP-aminohydrolase occurs directly in placental cytoplasm.

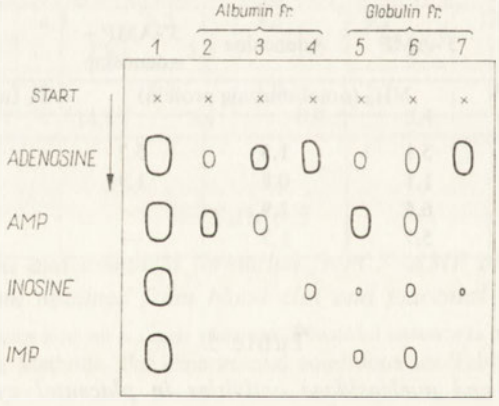

Fig. 1. Chromatographic separation of the nucleosides and nucleotides after incubation of 5′-AMP and adenosine with albumin and globulin fractions obtained from placental cytoplasm by dialysis. The albumin fraction (2.9 mg of protein) and globulin fraction (3.3 mg of protein) were incubated with 10 μmoles of adenosine or 5′-AMP as described in the text. After the indicated time, the reaction was stopped by placing the test tubes in a boiling water bath for 5 min. The samples were deproteinized by shaking with chloroform and 20 μl was applied on Whatman no. 1 paper and chromatographed as described in Methods. *1*, Mixture of standards. Albumin fraction and: *2*, AMP, 20 min; *3*, AMP, 40 min; *4*, adenosine, 40 min. Globulin fraction and: *5*, AMP, 20 min; *6*, AMP, 40 min; *7*, adenosine, 40 min.

DISCUSSION

Very little information exists about the metabolic pathways of purine and pyrimidine compounds in human placenta (Hagerman, 1969). It is well established that placenta contains 5′-nucleotidase (Ahmed & King, 1959; Cerletti, Fronticelli & Zichella, 1960) and adenosine aminohydrolase (Hayashi *et al.*, 1964). Thus in placenta ammonia may be produced from 5′-AMP through dephosphorylation to adenosine and subsequent deamination. An alternative pathway would be a direct deamination of 5′-AMP to 5′-IMP catalysed by AMP-aminohydrolase, as it is the case in many animal tissues.

Hayashi & Olmsted (1965) using ^{14}C-labelled 5′-AMP as substrate have come to the conclusion that AMP-aminohydrolase is absent from human placenta and uterus. Their procedure, however, involved 18 h of dialysis of placental extract against 0.01 M-tris buffer, pH 7.4, and this could have resulted in inactivation of AMP-aminohydrolase. It is well known that AMP-aminohydrolases from other tissues undergo rapid inactivation in solutions of low ionic strength and in absence of compounds containing -SH groups. To control their negative results with placenta and uterus, Hayashi & Olmsted (1965) used rabbit skeletal muscle as a reference tissue. This kind of control does not seem to be very fortunate as skeletal muscle has an exceptionally high activity of AMP-aminohydrolase exceeding many times the activity observed in other tissues (Purzycka, 1962). This means that the activity of the order observed in many animal tissues other than skeletal muscle could have been overlooked.

The results presented in this paper clearly indicate that AMP-aminohydrolase is present in human placenta. Not only 5′-AMP was a better source of ammonia than adenosine, when incubated with placental cytoplasm, but after dialysis the activities of AMP-aminohydrolase and adenosine aminohydrolase were partly separated. The results obtained with the cytoplasm after short and long homogenization indicate that these two enzymes are differently extracted from placental tissue. The direct deamination of 5′-AMP was confirmed by demonstration that in the conditions employed 5′-IMP was formed during incubation. The occurrence of a considerable amount of 5′-IMP in fresh placental tissue has been demonstrated by Cerletti, Fronticelli & Lauricella (1959).

The work reported in this paper has been partly supported by the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences. The authors wish to express their gratitude to dr. habil. Leon Żelewski for helpful discussion in the course of this work.

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AMP-AMINOHYDROLAZA ŁOŻYSKA LUDZKIEGO

Streszczenie

Badano dezaminację i defosforylację 5′-AMP oraz dezaminację adenozyny przez cytoplazmę łożyska ludzkiego. Stwierdzono, że cytoplazma łożyska uwalnia więcej amoniaku z 5′-AMP aniżeli z adenozyny. Aktywność wobec obu substratów częściowo rozdzielono dializując cytoplazmę wobec wody. Chromatografią na bibule wykazano powstawanie 5′-IMP z 5-′AMP. Otrzymane wyniki wskazują, że cytoplazma łożyska (praktycznie nie zanieczyszczona krwią) obok aminohydrolazy adenozyny zawiera swoistą aminohydrolazę 5′-AMP (EC 3.5.4.6).

Received 2 November, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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PHOSPHODIESTERASE II ACTIVITY AGAINST NUCLEOSIDE-3′ PHOSPHATE ESTERS AND SOME 5′-*O*-SUBSTITUTED ANALOGUES; AND AN IMPROVED RIBONUCLEASE SUBSTRATE, α-NAPHTHYL 5′-*O*-METHYLURIDINE-3′ PHOSPHATE

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1) The α-naphthyl and 5-bromo-4-chloro-3-indolyl esters of thymidine-3′ phosphate are hydrolysed by phosphodiesterase II (either in the purified form or in spleen extracts) at 0.5 - 1.0% of the rate for the *p*-nitrophenyl ester, which limits their applicability as histochemical substrates. 2) Phosphodiesterase II was shown to slowly hydrolyse α-naphthyl uridine-3′ phosphate, thus potentially interfering with the specificity of this substrate for ribonuclease. Various 5′-*O*-substituted nucleoside-3′ phosphate esters were therefore synthesized and tested for resistance to phosphodiesterase II. 3) Resistance to phosphodiesterase II was found to depend on the nature of the substituent. Almost absolute resistance was conferred on *p*-nitrophenyl and α-naphthyl esters of thymidine-3′ phosphate by introduction of a 5′-*O*-tetrahydropyranyl substituent. 5′-*O*-Acetylation of these compounds reduced the rate of their hydrolysis approximately 100-fold. 5′-*O*-Methyl substitution of α-naphthyl uridine-3′ phosphate led to a 15-fold reduction in the rate of hydrolysis of this substrate by the RNase-free phosphodiesterase II. 4) The various 5′-*O*-substituted derivatives were evaluated with regard to ease of preparation, stability, and resistance to tissue enzymes. An examination was also made of the effect of 5′-*O*-methylation and 5′-*O*-tetrahydropyranylation of α-naphthyl uridine-3′ phosphate on susceptibility to ribonuclease. The foregoing indicated that the α-naphthyl ester of 5′-*O*-methyluridine-3′ phosphate is a more suitable ribonuclease substrate with considerable resistance towards phosphodiesterase II. 5) The rates of hydrolysis by ribonuclease and phosphodiesterase II of α-naphthyl uridine-3′ phosphate and its 5′-*O*-methyl analogue were measured and compared with the rates of hydrolysis of the optimal enzymic substrates, RNA and 3′-terminated oligonucleotides, respectively. 6) The necessity for a phosphodiesterase II resistant ribonuclease substrate is discussed in relation to the ratio of the two enzyme activities in various tissues. 7) α-Naphthyl nucleoside-3′ phosphates and their 5′-substituted derivatives were shown to inhibit the hydrolysis of *p*-nitrophenyl thymidine-3′ phosphate by phosphodiesterase II.

PDase II[1] is known to occur in a variety of mammalian tissues (Razzell, 1961) and has been localized in the lysosomal fraction of rat liver (Van Dyck & Wattiaux, 1968; Erecińska, Sierakowska & Shugar, 1969). The enzyme is conveniently assayed against Tp-nitrophenyl, a substrate hydrolysed at a rate comparable to those for the natural substrates, the 5′-hydroxyl terminated oligonucleotides (Razzell & Khorana, 1961). Attempts were consequently made to develop a histochemical technique for PDase II, based on azo-dye coupling, and involving the use of another thymidine phosphate ester, Tp-naphthyl, as substrate. However, the latter compound proved ineffective due to its apparent resistance to PDase II (Sierakowska, Szemplińska & Shugar, 1963). Subsequently a histochemical procedure for PDase II was described with the use of 5-bromo-4-chloro-3-indolyl thymidine-3′ phosphate (Wolf, Horwitz, Freisler, Vazquez & Von der Muehll, 1968), but the associated indigogenic method proved to be relatively insensitive.

The difficulties encountered in PDase II localization by means of the foregoing compounds prompted us to re-examine the behaviour of this enzyme towards some pyrimidine nucleotide esters. This led to the finding of relatively low PDase II activity against naphthyl ribonucleotide esters which, in turn, might be expected to limit the specificity of the previously described assay for alkaline ribonuclease with Up-naphthyl (Zan-Kowalczewska, Sierakowska & Shugar, 1966). Consequently several 5′-*O*-substituted nucleotide-3′-esters were prepared and examined for resistance to PDase II, with a view to increasing the specificity towards RNase of 5′-*O*-substituted ribonucleotide 3′-esters. The possible utility of this approach was suggested by the fact that 5′-phosphate terminated oligonucleotides are resistant to PDase II (Razzell & Khorana, 1961), whereas a 5′-pyrophosphate substituted pyrimidine nucleoside-2′,3′-cyclic phosphate is susceptible to RNase (Michelson, 1961).

MATERIAL AND METHODS

Chromatography involved the use of Whatman no. 3 paper for preparative purposes and Merck TLC-plates Cellulose F for analytical purposes. The solvent systems used were:

A, *n*-butyl alcohol - water (86:14, v/v); *B*, ethyl alcohol - 1 M-ammonium acetate, pH 7.6 (5:2, v/v); *C*, *n*-butyl alcohol - acetic acid - water (5:2:3, by vol.); *D*, *n*-butyl alcohol - water - NH_4OH (86:14:1, by vol.); *E*, saturated aqueous ammonium sulphate - 1 M-sodium acetate, pH 7.6 - isopropyl alcohol (80:18:2, by vol.).

Tp-nitrophenyl, ammonium salt, was a product of Raylo Chemicals, Ltd. (Edmonton, Canada).

5′-O-Tetrahydropyranyl-Tp-nitrophenyl was obtained by treating 0.1 mmole of Tp-nitrophenyl (free acid) in 2 ml dry dioxane and 0.5 ml dimethyl sulfoxide with

[1] The following abbreviations are used: PDase II, phosphodiesterase II (EC 3.1.4.1); RNase, ribonuclease (EC 2.7.7.16); Tp-nitrophenyl, *p*-nitrophenyl thymidine-3′ phosphate; Tp-naphthyl, *α*-naphthyl thymidine-3′ phosphate; Up-naphthyl, *α*-naphthyl uridine-3′ phosphate.

0.6 ml trifluoroacetic acid and 0.3 ml dihydropyran under anhydrous conditions at room temperature for 3 hours; 2.5 ml conc. ammonia was then added to the cooled mixture and the total taken to dryness under reduced pressure with addition of triethylamine. The residue was dissolved in water, made up to pH 9 with ammonia, extracted with ether and again taken to dryness. The residue was dissolved in methanol and chromatographed on Whatman no. 3 with solvent *D*. The product (R_F 0.40) was eluted with methanol made alkaline with a few drops of NH_4OH. The yield was 75% of theoretical.

5'-O-Acetyl-Tp-nitrophenyl was prepared from Tp-nitrophenyl according to the procedure of Razzell & Khorana (1959). The product was chromatographed on Whatman no. 3 with solvent *C*, and 5'-*O*-acetyl-Tp-nitrophenyl (R_F 0.72) eluted with methanol, taken to dryness, dissolved in water and converted to the ammonium salt by passing through a column of Amberlite IRC 50 (NH_4^+ form). The overall yield of the product (R_F 0.39 on Merck TLC-plates Silica Gel F_{254} developed in 20% methanol in chloroform, as compared to R_F 0.22 for Tp-nitrophenyl) was 60% of theoretical.

Tp-naphthyl was prepared from 5'-*O*-tritylthymidine according to Sierakowska *et al.* (1963). Following phosphorylation the reaction mixture was taken to dryness under reduced pressure. The residue was taken up in chloroform, extracted twice with 1 M-triethylammonium bicarbonate buffer, pH 8.0, and subsequently with water, then again taken to dryness. The resulting gum was dissolved in 20 ml of 80% acetic acid, heated under reflux for 20 min and taken to dryness under reduced pressure. The residue was suspended in 15 ml water and set aside for 18 h at 4°C. The crystalline precipitate of triphenylcarbinol was filtered off and the aqueous solution brought to dryness under reduced pressure. The residue was dissolved in methanol and chromatographed on plates coated with Merck Silica Gel GF_{254} developed in 50% methanol in chloroform. Tp-naphthyl (R_F 0.65) was eluted with methanol, taken to dryness, redissolved in water and passed through a column of Dowex 50 (Na^+ form). The sodium salt of the product was crystallized twice from 4 ml methanol containing a few drops of ether - acetone (2:1, by vol). The yield was 75% of theoretical; λ_{max} (pH 7) 271 mμ, ε_{max} 1.44×10^4.

5'-O-Tetrahydropyranyl-Tp-naphthyl was prepared by treating 0.2 mmole dry Tp-naphthyl (free acid) dissolved in 4 ml of dry dioxane with 40 mg *p*-toluenesulphonic acid monohydrate and 0.5 ml dihydropyran under anhydrous conditions at room temperature for 24 hours. The reaction was terminated by adding 4 ml of concentrated ammonia to the cooled reaction mixture. The total was evaporated to dryness, dissolved in 10 ml water and extracted with ether. The aqueous solution was concentrated and chromatographed twice in the same direction on plates coated with Merck Aluminium oxide PF_{254} developed in 50% aqueous methanol. The band with an R_F 0.55 was eluted with 50% aqueous methanol, taken to dryness, dissolved in water and converted to the sodium salt by passing through a column of Dowex 50 (Na^+ form). The overall yield of the product (R_F 0.60 in solvent *A*) was 55% of theoretical.

5'-O-Acetyl-Tp-naphthyl was prepared according to the procedure of Razzell & Khorana (1959) by treatment of 150 mg of the dry pyridinium salt of Tp--naphthyl in 7.5 ml dry pyridine with 3.75 ml of freshly distilled acetic anhydride for 18 h at room temperature. The product was isolated by chromatography on Whatman no. 3 with solvent *C*. 5'-*O*-Acetyl-Tp-naphthyl (R_F 0.77) was eluted with methanol, taken to dryness, dissolved in water and converted to the sodium salt by passing through Dowex 50 (Na^+ form). The overall yield of the product (R_F 0.2 on Merck TLC-plates Silica Gel F_{254} developed in 20% methanol in chloroform) was 60% of theoretical.

Up-naphthyl and *5'-O-methyl-Up-naphthyl* were prepared according to procedures described elsewhere (Kole & Sierakowska, 1971).

3'(5')-O-Tetrahydropyranylthymidine was prepared by treating 1 mmole dry thymidine dissolved in 2.5 ml dry dimethylformamide and 1ml trifluoroacetic acid with 0.5 ml dry dihydropyran at room temperature under anhydrous conditions for about 20 min. The reaction mixture was evaporated under reduced pressure at below 30°C for several minutes, cooled, and 2.5 ml conc. ammonia added. The total was taken to dryness under reduced pressure and chromatographed on plates coated with Merck Aluminium oxide PF_{254} developed in 10% methanol in chloroform. The first band (R_F 0.5) formed above traces of unreacted thymidine (R_F 0.1) was eluted with 50% aqueous methanol, evaporated to dryness, taken up in water, extracted with ether and lyophilized. The overall yield of the product (R_F 0.20 in solvent *E*) was 15% of theoretical.

5-Bromo-4-chloro-3-indolyl thymidine-3' phosphate was generously contributed by Dr. J. P. Horwitz, Detroit Institute of Cancer Research.

Spleen PDase II was a Worthington (Freehold, N.J., U.S.A.) preparation, stabilized in solution according to Bernardi & Bernardi (1968). For 5'-O-acetyl substituted compounds and for the uridine derivatives, the RNase- and esterase-free PDase II (Bernardi & Bernardi, 1968; Bernardi & Cantoni, 1969), generously contributed by Dr. G. Bernardi, was employed. Rat spleen supernatant used as a source of PDase II was prepared from rat spleen homogenized in 10 volumes of 1 mM--EDTA, frozen-thawed 10 ×, centrifuged at 100,000 *g* for 1 hour and concentrated 20-fold by lyophilization.

PDase II activity was assayed against 10 mM substrates in 0.15 M-ammonium acetate, pH 5.7, 10 mM-EDTA and 0.1% Tween 80. The liberated *p*-nitrophenol was estimated according to Razzell & Khorana (1961), and liberated naphthol by the following modification of the procedure of Seligman & Nachlas (1949): Incubation of the 0.05 ml sample was terminated by addition of 1 ml of 0.2 M-sodium acetate buffer, pH 5.2, followed by 0.25 ml of a freshly prepared filtered 0.75% aqueous solution of Fast Garnet GBC (George Gurr, London). The mixture was shaken and the colour allowed to develop for 3 min. Subsequently 0.25 ml of 40% trichloroacetic acid was added with shaking and, after 5 min, the dye was extracted into 1.5 ml ethyl acetate. The tubes were cooled and the ethyl acetate layer was photometered at 540 mμ. Alternatively the course of hydrolysis was followed by chromatography on Merck TLC-plates cellulose F developed in solvent systems *A*, *B* or *E*.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

For histochemical purposes formol-calcium fixed or fresh frozen sections were incubated for 3 hours at room temperature with Tp-naphthyl, 2 mg/ml in 0.15 M--ammonium acetate buffer, pH 5.7, 10 mM-EDTA, 0.1% Tween 80 and Fast Garnet GBC, 1 mg/ml, or hexazonium pararosaniline according to Barka & Anderson (1962). The incubation medium was renewed every 30 min. In some experiments 0.1 M-acetate buffer, pH 5.9, without EDTA or Tween, was employed. Alternatively, sections were incubated for 4 - 24 h at room temperature and at 37°C with 5-bromo--4-chloro-3-indolyl thymidine-3′ phosphate, 1 mg/ml in 0.15 M-ammonium acetate buffer, pH 5.7, 10 mM-EDTA and 0.1% Tween 80. Occasionally, 0.1 M-acetate buffer, pH 5.0 or 5.9, without EDTA or Tween 80, was used.

RESULTS AND DISCUSSION

As shown in Tables 1 and 2, replacement of the *p*-nitrophenyl moiety by naphthyl in thymidine-3′ phosphate leads to a 150-fold decrease in the rate of hydrolysis by purified PDase II (Table 1) and by spleen supernatant (Table 2).

Table 1

Rates of hydrolysis of thymidine-3′ phosphate esters by purified spleen phosphodiesterase II

Commercial spleen PDase II was used routinely; for 5′-*O*-acetyl derivatives, Up-naphthyl and 5′-*O*-methyl-Up-naphthyl, an esterase- and RNase-free PDase II was employed. For the latter enzyme the rate of Tp-nitrophenyl hydrolysis was used as a point of reference to recalculate all the values and express them in units corresponding to those for the commercial enzyme. Hence the values for the RNase-free enzyme do not reflect its absolute specific activity but merely illustrate the relative rates towards the various compounds. Activities are in μmoles substrate hydrolysed per mg protein per hour at 37°. Rates of hydrolysis were determined at 10 mM substrate concentrations in 60 min incubations. For 5′-*O*-tetrahydropyranyl-Tp-esters the rates are overestimated due to spontaneous cleavage of tetrahydropyranyl groups, as explained in the text.

Substrate	Rate (μmol/h/mg protein)	V_{max} (μmol/h/mg protein)	$K_m \times 10^3$ (M)
Tp-nitrophenyl	350	400	1.4
5′-*O*-Tetrahydropyranyl--Tp-nitrophenyl	<0.1	—	—
5′-*O*-Acetyl-Tp-nitrophenyl	3.6	4	2.9
Tp-naphthyl	2.6	2.8	2.1
5′-*O*-Tetrahydropyranyl--Tp-napthyl	<0.1	—	—
5′-*O*-Acetyl-Tp-naphthyl	0.04	—	—
Up-naphthyl	1	1	1.6
5′-*O*-Methyl-Up-naphthyl	0.07	—	—

Table 2

Rates of hydrolysis of thymidine-3′ phosphate esters by rat spleen supernatant

Activities are in μmoles substrate hydrolysed per mg protein per hour at 37°. Rates of hydrolysis were determined at 10 mM substrate concentrations in 60 min incubations. In the case of 5′-*O*--tetrahydropyranyl-Tp-nitrophenyl, more concentrated spleen extracts give excessively high blank values which interfere with the assay of *p*-nitrophenol. For 5′-*O*-tetrahydropyranyl-Tp-naphthyl the rates are overestimated due to spontaneous cleavage of the 5′-*O*-tetrahydropyranyl group, as explained in the text. Rates for 5′-*O*-acetyl diesters are due to esterase activity in the spleen supernatant, as discussed in the text.

Substrate	Rate (μmol/h/mg protein)	V_{max} (μmol/h/mg protein)	$K_m \times 10^3$ (M)
Tp-nitrophenyl	7.0	8.7	1.2
5′-*O*-Tetrahydropyranyl--Tp-nitrophenyl	⩽0.05	—	—
5′-*O*-Acetyl-Tp-nitrophenyl	1.3	—	—
Tp-naphthyl	0.03	0.04	2.1
5′-*O*-Tetrahydropyranyl--Tp-naphthyl	<0.001	—	—
5′-*O*-Acetyl-Tp-naphthyl	0.02	—	—

The enormous difference in rates of hydrolysis of the naphthyl and *p*-nitrophenyl derivatives readily accounts for the histochemically observed resistance of Tp--naphthyl to PDase II (Sierakowska *et al.*, 1963). The naphthyl derivative is split only by excessive doses of the purified enzyme; and a minimum 1-hour incubation period with concentrated spleen supernatant is required for release of microgram quantities of naphthol. Several lines of evidence suggest that Tp-naphthyl hydrolysis by the spleen is indeed due to lysosomal PDase II: (1) the pH optimum is at pH 5 - 6; (2) formol-calcium fixed spleen sections incubated at pH 5 - 6 exhibit sites of enzyme activity while unfixed sections, subject to enzyme diffusion, do not; (3) enzyme activity is histochemically detectable following 3 hours incubation at room temperature in certain splenic cells, abundant in PDase II, but not in kidney and liver cells, poorer in enzyme activity.

The susceptibility of 5-bromo-4-chloro-3-indolyl thymidine-3′ phosphate to purified PDase II and spleen supernatant was estimated qualitatively by following chromatographically the rate of release of thymidine-3′ phosphate and found to approximate that of Tp-naphthyl. Difficulties in PDase II localization with Tp--naphthyl, resulting from relative substrate resistance, can consequently be expected to affect also the histochemical procedure based on the indigogenic principle. The latter, however, allows for prolonged incubation which, in the case of simultaneous azo-dye coupling, leads to decomposition of the diazotate. Thus, if prolonged incubations are acceptable, it may be possible to localize relatively active sites of PDase II by the indigogenic method.

The effect on PDase II activity of the *α*-naphthyl or 5-bromo-4-chloro-3-indolyl substituents is not altogether unexpected. The following observations indicate that PDase II is not indifferent to the ester substituent at the nucleoside 3′-phosphoryl: (a) Tp-nitrophenyl is hydrolysed at only 0.77 the rate for thymidylyl-(3′,5′)-thymidine-3′ phosphate (Razzell, 1961; Razzell & Khorana, 1961); (b) thymidylyl--(3′-3′)-deoxycytidine is resistant (Ohtsuka, Moon & Khorana, 1965); (c) 2,4-dinitrophenyl thymidine-3′ phosphate is hydrolysed 40% more rapidly than Tp-nitrophenyl (Tigerstrom & Smith, 1969).

The ability to demonstrate sites of exceptionally high PDase II activity with Tp-naphthyl is, however, a potential source of error in the assay and localization of RNase with the analogous ribonucleotide ester, Up-naphthyl. The latter compound is hydrolysed by PDase II at about 50% the rate for Tp-naphthyl (Table 1). It therefore seemed worthwhile to investigate the possibility of eliminating the threat of PDase II interference in RNase assays requiring prolonged incubations, by rendering Up-naphthyl resistant to PDase II.

Substitution of the terminal 5′-*O*-position of oligonucleotides by phosphate has been reported as absolutely inhibitory to PDase II activity (Razzell & Khorana, 1961). Since the phosphate group is subject to rapid removal by the ubiquitous tissue phosphomonoesterases, the susceptibility to PDase II following 5′-*O*-substitution with other groups was next investigated.

Table 1 shows the effect of tetrahydropyranyl, acetyl and methyl 5′-*O*-substituents of *p*-nitrophenyl and *α*-naphthyl nucleoside-3′ phosphates on their rates of hydrolysis by purified PDase II.

The tetrahydropyranyl group confers almost total resistance to PDase II. The rates of hydrolysis given in Table 1 for 5′-*O*-tetrahydropyranyl-Tp-esters are actually an overestimate. As demonstrated by thin-layer chromatography, they are due largely to traces of Tp-nitrophenyl and Tp-naphthyl resulting from spontaneous depyranylation during incubation at pH 5.7.

The higher rate of hydrolysis of 5′-*O*-acetyl-Tp-nitrophenyl is really due to the lesser resistance conferred by acetyl substitution of the 5′-*O*-position. It cannot possibly be attributed to the presence of traces of Tp-nitrophenyl since 5′-*O*-acetylthymidine-3′ phosphate constitutes the *sole* product of hydrolysis with the esterase--free PDase II.

The 5′-*O*-methyl group confers lesser resistance to PDase II than the acetyl. 5′-*O*-Methyl-Up-naphthyl is split by the RNase-free enzyme at 7% the rate for Up-naphthyl.

Table 2 confirms in general the inhibitory effect of 5′-*O*-substitution on the rates of hydrolysis of Tp-nitrophenyl and Tp-naphthyl by rat spleen supernatant.

5′-*O*-Tetrahydropyranyl substitution effectively reduces the substrate's susceptibility to the supernatant enzyme. The specificity of this effect is further testified to by the fact that removal of the tetrahydropyranyl group from 5′-*O*-tetrahydropyranyl-Tp-naphthyl by treatment with acetic acid restores the substrate's initial susceptibility to the supernatant enzyme.

The inconsistencies in the behaviour of 5′-*O*-acetylthymidine 3′-phosphate esters are due to removal of the 5′-*O*-acetyl by esterases present in the supernatant prior to hydrolysis by PDase. This is substantiated by (a) the finding of activity against 5′-*O*-acetylthymidine in the spleen supernatant and (b) the fact that thymidine rather than 5′-*O*-acetylthymidine is the product of hydrolysis of 5′-*O*-acetyl-Tp--naphthyl by the spleen supernatant.

The foregoing, and other considerations, indicate that a suitable 5′-*O*-protective group providing resistance to PDase II in a ribonuclease substrate should combine the following properties: (a) insure sufficient steric hindrance towards PDase II; (b) good solubility in aqueous media; (c) resistance to tissue enzymes and stability in media buffered to pH 5 - 9; (d) stability during removal of the 2′-*O*-protecting group, which is the final step in the preparation of the substrate; (e) exert little effect on substrate susceptibility to RNase.

The 5′-*O*-tetrahydropyranyl group satisfies the first two requirements. It is reasonably stable in slightly acidic media and inert to tissue enzymes, e.g. the tetrahydropyranyl group of 3′(5′)-*O*-tetrahydropyranylthymidine is not removed during overnight incubation at 37°C with 2% kidney homogenate or 4% homogenates of pancreas, liver and duodenum both in 0.08 M-tris-HCl buffer, pH 8.0, and in 0.08 M-sodium acetate buffer, pH 5.2. Furthermore, the presence of the 5′-*O*-tetrahydropyranyl group decreases the rate of hydrolysis of Up-naphthyl by crystalline pancreatic RNase by only 40%.

However, 5′-*O*-tetrahydropyranyl-Up-naphthyl cannot be obtained in quantitative yield by controlled acidification of 2′,5′-di-*O*-tetrahydropyranyl-Up-naphthyl, the stable intermediate in Up-naphthyl synthesis. Simultaneous partial removal of the 5′-*O*-tetrahydropyranyl group occurs, necessitating isolation of 5′-*O*-tetrahydropyranyl-Up-naphthyl by supplementary chromatographic procedures with some concomitant decomposition of this relatively labile compound.

The acetyl group confers pronounced resistance to PDase II but is unsatisfactory because of its susceptibility to tissue esterases, as might have been anticipated. In fact, enzymic removal of acetyl protecting groups has been proposed as a useful step in organic synthesis (Zan-Kowalczewska *et al.*, 1966; Sachdev & Starovsky, 1969).

The 5′-*O*-methyl substituent decreases only 15-fold the susceptibility of Up--naphthyl to PDase II. It is, however, stable at *all* pH values, hence also during removal of the 2′-*O*-protecting group. It remains intact following overnight incubations with 5% homogenates of rat brain, kidney, liver, spleen and pancreas in 0.1 M-acetate buffer, pH 5.2, and 0.1 M-phosphate buffer, pH 8.0. Furthermore, it decreases the rate of hydrolysis by RNase by only 40%. Hence, 5′-*O*-methyl--Up-naphthyl appears to be an acceptable RNase substrate, significantly superior to Up-naphthyl. Because of the chemical and enzymic stability of the 5′-*O*-methyl linkage, steps are under way to synthesize analogous potential substrates with higher alkyl groups.

At first glance the utility of Up-naphthyl as an RNase substrate does not seem affected by its susceptibility to PDase II, since its rate of hydrolysis by RNase is

5 mmol/h/mg protein and that by PDase II is only 1 μmol/h/mg protein. However, when one compares the rates of hydrolysis of Up-naphthyl by these enzymes to the rates of hydrolysis of their respective "natural" optimal substrates, these differences largely diminish and Up-naphthyl appears relatively resistant to both enzymes.

PDase II hydrolyses its optimal substrate, 3′-phosphate terminated oligonucleotides, at approximately 1.3 times the rate for Tp-nitrophenyl, which in turn is hydrolysed about 350 times more rapidly than Up-naphthyl. Thus the rate of hydrolysis of Up-naphthyl equals 0.22% that of the optimal PDase II substrate. On the other hand, Up-naphthyl is hydrolysed by pancreatic RNase at a rate of 5 mmol/mg/h as compared to 500 mmol/mg/h for RNA, i.e. at 1% the rate for the optimal RNase substrate.

This gives a ratio of RNase to PDase II activity *vs.* Up-naphthyl equal to 1% of the optimal rate for RNase to 0.22% of the optimal rate for PDase II; i.e. only a 4.5-fold greater "effective" susceptibility of Up-naphthyl to RNase. This difference in "effective" susceptibility is sufficient for successful RNase assays in several mammalian tissues where the favourable ratio of RNase to PDase II activity overcomes the relatively small difference in susceptibility. Such a ratio of RNase to PDase II activity may not, however, always hold. In such cases the use of 5′-*O*-methyl-Up-naphthyl, hydrolysed by RNase at a rate about 40 times its "effective" rate of hydrolysis by PDase II, will ensure higher specificity of RNase estimations.

The hydrolysis of Tp-nitrophenyl by PDase II is inhibited by *α*-naphthyl nucleoside-3′ phosphates and their 5′-substituted derivatives listed in Table 1. 5′-*O*-Tetrahydropyranyl-Tp-naphthyl is the most effective of these inhibitors and its effectiveness is about equal to that of thymidine-3′ phosphate.

We are deeply indebted to Dr. G. Bernardi for his purified phosphodiesterase II; to Dr. J. P. Horwitz for a sample of the indolyl substrate; to Mr. Ryszard Kole for some of the synthetic preparations; to Mr. Jarosław Kuśmierek for the 5′-*O*-methyluridine used for the preparation of the *α*-naphthyl ester; and to Mrs. Halina Szemplińska for excellent technical assistance. This investigation was supported by the Wellcome Trust, the World Health Organization and the Agricultural Research Service, U.S. Dept. of Agriculture.

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DZIAŁANIE FOSFODWUESTERAZY II NA ESTRY 3′-FOSFORANÓW NUKLEOZYDÓW I ICH 5′-PODSTAWIONE POCHODNE; 3′-*α*-NAFTYLOFOSFORAN 5′-*O*-METYLOURYDYNY — UDOSKONALONY SUBSTRAT DLA RYBONUKLEAZY

Streszczenie

1. Fosfodwuesteraza II (preparat oczyszczony lub enzym z supernatantu śledziony) hydrolizuje estry *α*-naftylowe i 5-bromo-4-chloro-3-indolilowe 3′-fosforanu tymidiny z szybkością wynoszącą 0.5 - 1.0% szybkości hydrolizy analogicznego estru *p*-nitrofenylowego.

2. Fosfodwuesteraza II hydrolizuje bardzo wolno 3′-*α*-naftylofosforan urydyny będący substratem dla rybonukleaz, co ogranicza jego swoistość. Z tego powodu dokonano syntezy szeregu estrów 3′-fosforanów nukleozydów o zablokowanej pozycji 5′ i zbadano ich wrażliwość na działanie fosfodwuesterazy II.

3. Oporność na działanie fosfodwuesterazy II zależy od rodzaju podstawnika w pozycji 5′ estrów 3′-fosforanów nukleozydów. Wprowadzenie podstawnika 5′-*O*-tetrahydropyranylowego powoduje niemal całkowitą oporność estrów *p*-nitrofenylowych i *α*-naftylowych 3′-fosforanów tymidyny na działanie fosfodwuesterazy II. Obecność grupy 5′-*O*-acetylowej zmniejsza 100-krotnie szybkość hydrolizy tych związków. Wprowadzenie grupy 5′-*O*-metylowej do 3′-*α*-naftylofosforanu urydyny powoduje już tylko 15-krotne zmniejszenie szybkości hydrolizy tego substratu przez pozbawioną RNazy fosfodwuesterazę II.

4. Dokonano oceny szeregu podstawników w pozycji 5′ z punktu widzenia łatwości uzyskania i trwałości produktu oraz oporności podstawnika na działanie enzymów tkankowych. Zbadano wpływ grupy metylowej i tetrahydropyranylowej w pozycji 5′ 3′-*α*-naftylofosforanu urydyny na szybkość hydrolizy pod wpływem rybonukleazy. Stwierdzono, że 3′-*α*-naftylofosforan 5′-*O*-metylourydyny jest zadowalającym substratem dla rybonukleazy, wykazującym znaczną oporność na działanie fosfodwuesterazy II.

5. Porównano szybkość hydrolizy 3′-*α*-naftylofosforanu urydyny oraz jego 5′-*O*-metylowego analogu pod wpływem rybonukleazy i fosfodwuesterazy II z szybkością hydrolizy optymalnych substratów dla tych enzymów, a mianowicie RNA oraz 3′-fosforanów oligonukleotydów.

6. Rozpatrzono przydatność substratu dla rybonukleaz, opornego na działanie fosfodwuesterazy II, z punktu widzenia względnych aktywności obu enzymów w różnych tkankach.

7. Stwierdzono, że 3′-*α*-naftylofosforany nukleozydów i ich 5′ podstawione pochodne hamują działanie fosfodwuesterazy II na 3′-*p*-nitrofenylofosforan tymidiny.

Received 19 November, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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INTERACTION OF LUPIN ISOLEUCINE-tRNA SYNTHETASE WITH ISOLEUCINE TRANSFER RIBONUCLEIC ACID MEASURED BY THE THERMAL INACTIVATION METHOD

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Isoleucyl-tRNA synthetase from yellow lupin seeds is stabilized against thermal inactivation by isoleucine tRNA and isoleucine tRNA deprived of terminal adenosine. Kinetic analysis of the inactivation process indicates that the synthetase forms with $tRNA^{Ile}$ a 1:1 complex. The dissociation constant of the reaction is of 10^{-9} M magnitude at the temperature range of enzyme denaturation (45° - 48°C). Formation of the complex at this temperature range is exergonic (-13 kcal/mol) and exothermic (-64 kcal/mol). It occurs with a large decrease in enthropy (-0.16 kcal/mol/degree). The use of the thermal inactivation method for investigation of the aminoacyl-tRNA synthetase-nucleic acid interaction is discussed.

In a recent paper from this laboratory it was reported that isoleucyl-tRNA synthetase from yellow lupin seeds was protected against heat inactivation by its isoleucine tRNA (Kędzierski & Pawełkiewicz, 1970).

The increased stability of the enzyme is attributed to the specific and reversible interaction between the synthetase and tRNA with the formation of the complex that is more stable than the free enzyme.

Kinetic analysis of the inactivation process enables to calculate the thermodynamic parameters of the complex formation assuming that the dissociation constant, k_2/k_1, of the reversible reaction:

$$\text{enzyme} + \text{tRNA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{enzyme-tRNA}$$

is identical with the protection constant, K_p (Chuang, Atherly & Bell, 1967). The latter was defined by Burton (1951), in analogy to the Michaelis-Menten constant, as the concentration of substrate that provides half-maximal protection of the enzyme against thermal inactivation under given conditions.

In this report, thermodynamic data for the formation of isoleucine-tRNA synthetase - tRNA complex are given, based on the above assumption. A preliminary communication was presented at the 8th Meeting of the Polish Biochemical Society, Szczecin, May 1970.

MATERIALS AND METHODS

L-[U-^{14}C]Isoleucine, 79.3 mCi/mmol, was obtained from the Institute for Research, Production and Application of Radioisotopes (Praha, Czechoslovakia); ATP was from Carl Roth (Karlsruhe, West Germany) and was neutralized with tris before use; 2-mercaptoethanol was from Koch-Light Lab. Ltd. (Colnbrook, Bucks., England); 2,5-diphenyloxazole (PPO) was from Reanal (Budapest, Hungary) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) from Calbiochem. (Los Angeles, Calif., U.S.A.). Yellow lupin seed tRNA was isolated as described by Legocki, Szymkowiak, Wiśniewski & Pawełkiewicz (1970) and the crude preparation was partially purified by chromatography on DEAE-cellulose at 72°C according to Legocki, Szymkowiak & Pawełkiewicz (1968) and contained about 14% of tRNA^{Ile}. The latter preparation was used in kinetic experiments.

tRNA deprived of terminal adenosine (tRNApCpCp) was prepared as follows: to 10 mg of purified lupin tRNA dissolved in 1 ml of 0.1 M-sodium acetate, pH 5, 0.6 ml of 0.05 M-$NaIO_4$ was added and the mixture was incubated at 25°C for 30 min in the dark. Then 0.5 ml of 2.5 M-NaCl was added and the oxidized tRNA was precipitated with 2.5 volumes of ethanol and collected by centrifugation after standing 1 h at -15°C. The presence of $NaIO_4$ in the supernatant was checked by measuring the decrease in absorption at 232 mμ on addition of ethylene glycol. The tRNA precipitate was dissolved in 1 ml of water and the remaining traces of $NaIO_4$ destroyed with 0.2 ml of 1% glucose on the 10 min incubation at 25°C. tRNA was reprecipitated with ethanol in the presence of sodium chloride and subjected to hydrolysis in 0.05 M-lysine-HCl buffer, pH 8.0, at 45°C for 90 min according to Baldwin & Berg (1966) in order to remove the terminal oxidized nucleoside. tRNApCpCp was precipitated twice with ethanol from 0.02 M-sodium acetate buffer, pH 5.0, and finally dialysed against water. The solution of the tRNA preparation obtained, completely deprived of acceptor activity for isoleucine, was kept frozen at -15°C.

Isoleucyl-tRNA synthetase was isolated and purified as described by Kędzierski & Pawełkiewicz (1970). All the kinetic studies reported were carried out with a single enzyme preparation.

Assay of the isoleucyl-tRNA synthetase activity and thermal inactivation. The reaction mixture contained in a final volume of 100 μl: 10 μmoles of tris-HCl buffer, pH 7.5; 1 μmole of $MgCl_2$; 2 μmoles of 2-mercaptoethanol; 0.1 mg of enzyme preparation and tRNA (0 - 0.2 mg). Before thermal inactivation the mixture was always preincubated for 5 min at 30°C and then heated at given temperature in a thermostatically controlled water bath for the indicated time. Temperature variations during the incubation period did not exceed 0.1°C. After incubation the sample was chilled in ice-cold water and to determine the remaining synthetase activity, 30 μl of a solution containing 1 μmole of ATP, 3 μmoles of [^{14}C]isoleucine and 0.2 mg of tRNA was added; after incubation for 20 min at 37°C, 100 μl of the reaction mixture was applied to Whatman 3*MM* paper disc (Ø 25 mm) which was washed three times, for 15 min each, successively in 5% trichloroacetic acid solution, in ethanol - ether

(1:1, v/v), and finally in ether. The dried disc was placed in a vial containing 3 ml of liquid scintillator (4 g of PPO, 0.1 g of POPOP in 1 liter of toluene) and counted in the scintillation counter.

The control was not heated and the blank did not contain tRNA and was pre-incubated at 50°C for 15 min before the addition of ATP and [^{14}C]isoleucine.

Calculation of protection constant, K_p. Inactivation rate constant, k, was determined graphically from the plot of first-order kinetics according to equation:

$$\log a = -\frac{k}{2.3} t + 2$$

where a is the percentage of the remaining activity and t, time of inactivation in minutes. Protection constant K_p was determined graphically from the plot according to O'Sullivan & Cohn (1966):

$$\frac{k}{k_0} = \frac{K_p}{c^n} \cdot \left(1 - \frac{k}{k_0}\right) + \frac{k_\infty}{k_0}$$

where k_0, k and k_∞ are the apparent first-order constants of enzyme inactivation determined in systems without tRNA, with tRNA in the given c concentration, and with tRNA in its saturating concentration, respectively; n is the number of moles of tRNA per enzyme molecule.

RESULTS

Kinetics of thermal inactivation of isoleucyl-tRNA synthetase. Inactivation of free enzyme followed the first-order kinetics (Fig. 1) and the inactivation rate constants

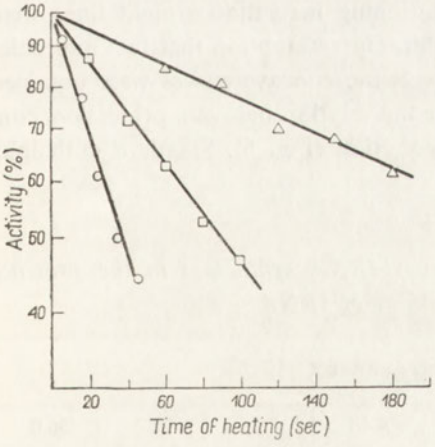

Fig. 1

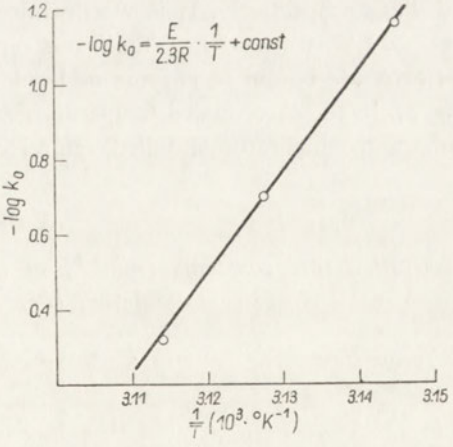

Fig. 2

Fig. 1. Thermal inactivation of isoleucyl-tRNA synthetase at 44.8° (△), 46.6° (□) and 48°C (○).

Fig. 2. Arrhenius plot for inactivation of isoleucyl-tRNA synthetase.

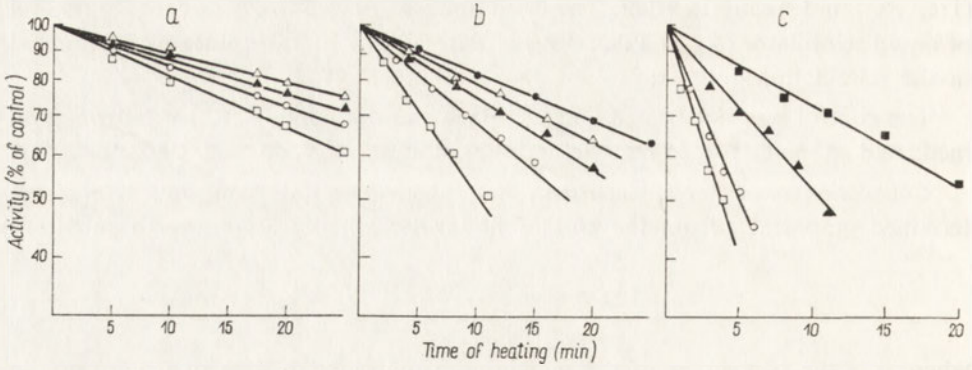

Fig. 3. Kinetics of isoleucyl-tRNA synthetase inactivation in the presence of tRNA at *a*, 44.8°; *b*, 46.6°; *c*, 48.0°C. Concentrations of tRNA are: 1.12×10^{-8} M (□), 2.24×10^{-8} M (○), 4.48×10^{-8} M (▲), 8.96×10^{-8} M (△), 2.24×10^{-7} M (●), 5.60×10^{-7} M (■).

were 0.07 min^{-1}, 0.20 min^{-1}, and 0.48 min^{-1} at the temperatures of 44.8°, 46.6° and 48°C, respectively. The reaction rates were beyond the range of experimental determination below or above 45° and 48°C, respectively. The obtained values fitted well the Arrhenius relation (Fig. 2). The activation energy of denaturation calculated from that equation was 120 kcal/mol.

Kinetics of thermal inactivation of isoleucyl-tRNA synthetase in the presence of isoleucine tRNA. When isoleucine tRNA was present during thermal reaction, the first-order kinetics of inactivation was retained. The inactivation rates were decreasing with increasing tRNA concentrations at each of the investigated temperatures (Fig. 3). Values of inactivation rate constants are given in Table 1. From these data protection constants of inactivation were determined graphically for three temperatures (Fig. 4). K_p were 0.8×10^{-9} M, 1.2×10^{-9} M and 2.2×10^{-9} M at 44.8°, 46.6° and 48°C, respectively. It is worthwhile mentioning here that straight lines were obtained only for $n=1$ in O'Sullivan-Cohn plot. On assumption that two molecules of tRNA are bound to enzyme molecule ($n=2$) the concave curves were obtained (Fig. 5). In the investigated temperature range the relation between protection constants and temperature fulfilled van't Hoff equation (Fig. 6). Standard enthalphy

Table 1

Inactivation rate constants (min^{-1}) of isoleucyl-tRNA synthetase in the presence of different concentrations of tRNA

Temperature of inactivation (°C)	tRNA concentration ($\times10^{-8}$ M)						
	0	1.12	2.24	4.48	8.96	22.4	56.0
44.8	0.07	0.0088	0.0067	0.0055	0.0050	—	—
46.6	0.20	0.028	0.017	0.012	0.010	0.0083	—
48.0	0.48	0.087	0.058	0.028	—	—	0.014

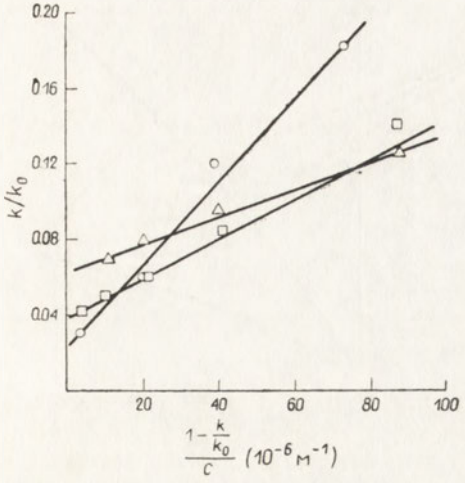

Fig. 4

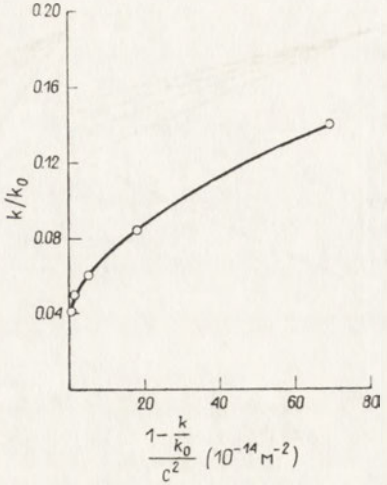

Fig. 5

Fig. 4. O'Sullivan-Cohn plot for inactivation of isoleucyl-tRNA synthetase at 44.8° (△), 46.6° (□) and 48.0°C (○) assuming that one molecule of tRNA is bound to the enzyme molecule.

Fig. 5. O'Sullivan-Cohn plot for inactivation of isoleucyl-tRNA synthetase at 46.6°C assuming that two molecules of tRNA are bound to the enzyme molecule.

change of the formation of the complex between isoleucyl-tRNA synthetase and $tRNA^{Ile}$, determined from Fig. 6, amounts to about -64 kcal/mol. Free energy changes for this reaction calculated according to equation: $F = RT1nK_p$ were -13.3, -13.1 and -12.7 kcal/mol at 44.8°, 46.6° and 48°C, respectively. Changes of entropy

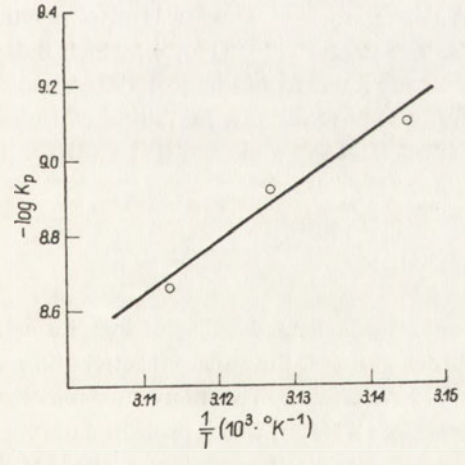

Fig. 6

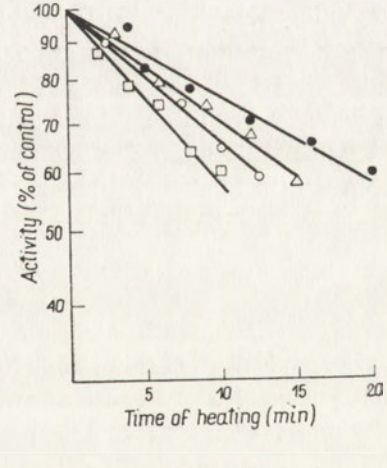

Fig. 7

Fig. 6. Van't Hoff plot of protection constants, K_p, of isoleucyl-tRNA synthetase protected by $tRNA^{Ile}$.

Fig. 7. Inactivation of isoleucyl-tRNA synthetase in the presence of tRNApCpCp in concentrations of 1.12×10^{-8} M (□), 2.24×10^{-8} M (○), 4.48×10^{-8} M (△), and 8.96×10^{-8} M (●).

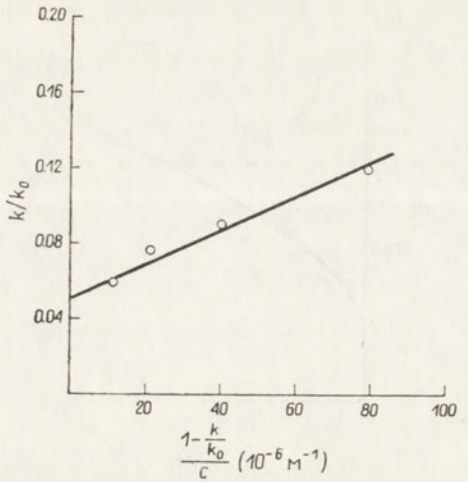

Fig. 8

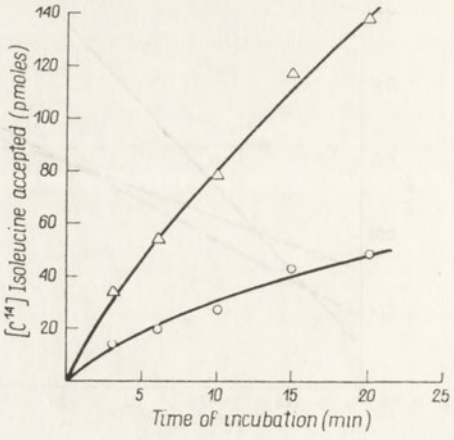

Fig. 9

Fig. 8. O'Sullivan-Cohn plot for inactivation of isoleucyl-tRNA synthetase at 46.6°C assuming that one molecule of tRNApCpCp is bound to the enzyme molecule.

Fig. 9. Effect of tRNApCpCp on kinetics of isoleucyl-tRNA formation. 8.4 μM-tRNA was aminoacylated with [^{14}C]isoleucine in the standard reaction mixture in the absence (△) or in the presence (○) of 8.4 μM-tRNApCpCp.

calculated from the classical relation: $\Delta H = \Delta F - T\Delta S$, were -0.16 kcal/mol/degree at each given temperature.

Kinetics of thermal inactivation of isoleucine-tRNA synthetase in the presence of tRNA^{Ile}pCpCp, tRNA lacking the 3′-terminal adenosine, i.e. unable to accept isoleucine, showed the same protection effects as tRNA^{Ile} in the inactivation experiments (Fig. 7). The affinity of tRNApCpCp to synthetase was also practically unchanged. Protection constant at 46.6°C was 0.9×10^{-9} M (Fig. 8), which indicates that adenylic acid residue either did not contribute to binding of tRNA molecule to the synthetase or that this contribution was insignificant. Inhibition of isoleucyl--tRNA synthesis in the presence of modified tRNA^{Ile} molecule was a further proof of tRNApCpCp binding to the synthetase (Fig. 9).

DISCUSSION

Isoleucine-tRNA synthetase from lupin seeds heated at 45°C lost its activity rapidly. The inactivation followed first-order kinetics, the apparent activation energy calculated from velocity constants was 120 kcal/mol. The first-order kinetics of inactivation was retained also in the presence of tRNA^{Ile}, which protected the enzyme specifically against thermal denaturation in concentration as low as 10^{-8} M. This protection could be observed experimentally in the investigated system in the narrow temperature range 45° - 48°C. Protection constants (K_p), considered by Chuang *et al.* (1967) as identical with dissociation constants of tRNA - enzyme complex, were calculated from inactivation rate constants in different tRNA^{Ile} concentrations

and at different temperatures. The calculated K_p approximated 10^{-9} M under the conditions used and the corresponding kinetic data were consistent with the assumption that the complex is composed of 1 mole of tRNA per mole of the synthetase.

Yarus & Berg (1967) found using the nitrocellulose filter retention technique the value of 10^{-8} M for K_p of *E. coli* isoleucine-tRNA synthetase. The corresponding values for valine-activating system obtained by Hélène, Brun & Yaniv (1969) by fluorescence spectroscopy varied from 10^{-5} to 10^{-8} M depending on $tRNA^{Val}$ species, pH and kind of buffer used. Yaniv & Gros (1969) applied the heat inactivation method for determination of K_p for genetically altered valyl-tRNA synthetase isolated from two thermosensitive *E. coli* mutants and obtained similar values (10^{-7} M).

Changes of thermodynamic function calculated for the formation of lupin isoleucyl-tRNA synthetase - $tRNA^{Ile}$ complex indicate that this process is exothermic ($\Delta H = -64$ kcal/mol) and exergonic ($\Delta F = -13$ kcal/mol). The low value for the change of free energy could suggest that the enzyme appears in plant cells always in the complex form with its specific tRNA. In this case, the aminoacylation process would not involve the aminoacyl-adenylate-enzyme intermediate considered in most of reaction mechanisms. This one-stage aminoacyl-tRNA formation was recently suggested by Loftfield & Eigner (1969), and by Marshall & Zamecnik (1970).

The large decrease of entropy for the complex formation (-0.16 kcal/mol/degree) indicates that the complex has a more ordered structure than the free components. It is often assumed that conformational changes result from the complex formation between a synthetase and its tRNA. Some more direct experimental proof of this idea was given by Ohta, Shimada & Imahori (1967) who showed by circular dichroism measurements that tyrosine-specific tRNA induced a decrease in helical content of the yeast tyrosyl-tRNA synthetase molecule.

Isoleucyl-tRNA synthetase of lupin is also protected against thermal inactivation by $tRNA^{Ile}$ deprived of terminal adenosine. The affinity of the modified molecule to the enzyme appears to be unchanged. This suggests that the terminal nucleoside has no effect on the formation and stability of the complex. This is consistent with the recent observation of Lagerkvist & Rymo (1970) that splitting off even 6 - 7 nucleotides from 3'-end of yeast $tRNA^{Val}$ does not abolish its ability to form a complex with valine synthetase as measured by the filtration method. However, the altered valyl-tRNA synthetase from thermosensitive *E. coli* mutants required the presence of intact tRNA molecule for stabilization of the enzyme against heating (Yaniv & Gros, 1969).

Our results indicate that the thermal inactivation method may be successfully applied to investigate the interaction between synthetase and nucleic acid. The advantage of the method lies in its simplicity. The experiments can be conducted even with relatively crude synthetase preparations, i.e. under more physiological conditions, where interaction between an enzyme and the other proteins may play some role in the investigated reaction. The method, however, requires elaboration of appropriate inactivation conditions. The lack of protection by tRNA in some systems may be due to the unsuitable experimental conditions. Makman & Cantoni

(1966), for example, found no effect of yeast $tRNA^{Ser}$ on stabilization of yeast seryl-tRNA synthetase at 47°C, although in our laboratory (Jakubowski, unpublished data) a system was developed in which the same enzyme was effectively protected against heat by yeast tRNA. The lack of tRNA response in stabilization of a synthetase may result also from a relatively high value of the dissociation constant at a given temperature of inactivation. The discussed method, however, is not free of some disadvantages. The process of inactivation occurs at high, unphysiological temperatures at which large conformational changes occur in protein and partly, at least, in tRNA as well. The protection of an enzyme against heat inactivation is itself a very complicated phenomenon. Besides specific protectors, some unspecific ones exert their influence on protein molecule, and a variety of conditions may alter the rates of inactivation. Yeast lysyl-tRNA synthetase was found to be thermolabile and the activity was completely destroyed on a 2 min heating at 40°C in the presence of 1 mM-Mg^{2+}. Sensitivity of the enzyme decreased, however, in the absence of Mg^{2+} (Berry & Grunberg-Manago, 1970). The same enzyme is protected against heat not only by tRNA but also by polyuridylic acid, and formation of the corresponding complexes between the enzyme and tRNA, or the enzyme and polyuridylic acid can be demonstrated. On the other hand, polycytidylic acid and polyadenylic acid do not form stable complexes although they stabilize the enzyme (Latendre, Humphreys & Grunberg-Manago, 1969; Berry & Grunberg-Manago, 1970). Thus, although the available evidence supports the view that the mechanism of protection involves the formation of specific complexes, the degree of this specificity varies from one protector to another.

The relation between tRNA binding and the effect on the catalytic sites of a synthetase was also under consideration. Mehler & Mitra (1967) showed specificity of requirement for $tRNA^{Arg}$ in the activation of arginine by arginyl-tRNA synthetase of *E. coli*. The $tRNA^{Arg}$ deprived of 3′-terminal nucleoside was not able to stimulate this reaction, although the modified tRNA molecule stabilized the enzyme against thermal inactivation (Mitra, personal communication cited by Yaniv & Gros, 1969). These and similar facts limit the validity of interpretation of results obtained exclusively by the thermal inactivation method. However, the importance of specific protein-nucleic acid interaction appears to be so great that all potentially useful techniques should be fully exploited.

This investigation was supported in part by the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

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WSPÓŁDZIAŁANIE IZOLEUCYLO-tRNA SYNTETAZY I IZOLEUCYNOWEGO tRNA, BADANE METODĄ TERMICZNEJ INAKTYWACJI ENZYMU

Streszczenie

tRNA specyficzny dla izoleucyny oraz tRNA pozbawiony końcowej adenozyny chroni przed ermiczną inaktywacją izoleucylo-tRNA syntetazę izolowaną z nasion łubinu żółtego. Analiza kinetyki procesu inaktywacji wykazuje, że syntetaza tworzy z $tRNA^{Ile}$ kompleks w stosunku molowym 1:1. Stała dysocjacji reakcji tworzenia kompleksu w granicach temperatur denaturacji enzymu (45° - 48°C) jest rzędu 10^{-9} M. Tworzenie kompleksu w temperaturze 45 - 48°C jest reakcją egzoergiczną (-13 kcal/mol) i egzotermiczną (-64 kcal/mol) i przebiega ze znacznym spadkiem entropii (-0.16 kcal/mol/stopień). W pracy przedyskutowano stosowanie metody inaktywacji termicznej w badaniu interakcji syntetaz z kwasami nukleinowymi.

Received 21 November, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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THE EFFECT OF HIGH SALT CONCENTRATION ON FIDELITY OF TRANSLATION BY *ESCHERICHIA COLI* RIBOSOMES

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1. Washing of *Escherichia coli* B ribosomes at high salt concentration (1 M-NH_4Cl--tris buffer) led to a considerable increase in ambiguity of poly U translation. 2. Storage of the washed ribosomes for a few days at 2°C in the same buffer restored the fidelity of translation. 3. The appearance and disappearance of ambiguous translation was demonstrated both in incorporation experiments and by binding of aminoacyl--tRNA's to a poly U-ribosome complex.

It has been previously demonstrated that *Escherichia coli* ribosomes are more resistant to factors causing ambiguity in recognition of poly U than the ribosomes from *Bacillus stearothermophilus* (Chomczyński, Perzyński & Szafrański, 1969; Perzyński, Chomczyński & Szafrański, 1969). The results of further experiments seem to suggest that differences in the behaviour of the ribosomes from these two sources are due to differences in structure (Szafrański, Perzyński, Chomczyński, Zagórska & Zwierzchowski, 1970).

In the present work it was demonstrated that the *E. coli* B ribosomes prepared in the presence of high salt concentration also showed a high ambiguity in poly U translation, and that this property disappeared on storage of the ribosomes. A preliminary account of this work has been published (Chomczyński & Szafrański, 1971).

MATERIALS AND METHODS

Chemicals were purchased from the following sources: L-[U-^{14}C]leucine (sp.act. 90 Ci/mol) and L-[U-^{14}C]phenylalanine (sp.act. 118 Ci/mol) from the Institute for Research, Production and Utilization of Radioisotopes (Prague, Czechoslovakia);

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L-[G-^{3}H]phenylalanine (sp.act. 500 Ci/mol) from the Radiochemical Centre (Amersham, Bucks., England); L-[^{12}C]amino acids, creatine phosphokinase and polyuridylic acid (potassium salt, mol.wt. 64 000) from Calbiochem (Los Angeles, Calif., U.S.A.); phosphocreatine, ATP, and GTP from Serva GmbH (Heidelberg, West Germany); deoxyribonuclease (ribonuclease-free) from Worthington (Freehold, N.J., U.S.A.); 2,5-diphenyloxazole (PPO), 1,4-bis(5-phenyloxazol 2-yl)-benzene (POPOP), hydroxide of hyamine 10-X from Packard Instrument Co. (Downers Grove, Ill., U.S.A.).

Buffer solutions used: TNM buffer (10 mM-tris, pH 7.4 - 70 mM-NH_4Cl - 14 mM-magnesium acetate - 6 mM-2-mercaptoethanol); 1 M-NH_4Cl-tris buffer (1 M-NH_4Cl - 10 mM-magnesium acetate - 10 mM-tris-HCl, pH 8.1); buffer P (70 mM-NH_4Cl - 10 mM-tris-HCl, pH 7.4, and varying concentrations, 5 - 40 mM, of magnesium acetate).

Culture of Escherichia coli *B.* This was carried out as described by Perzyński & Szafrański (1967). The bacteria were collected by centrifugation at the late exponential phase of growth.

Preparation of ribosomes and the 150 000 g *supernatant.* The bacteria frozen to -40°C were disrupted in an Eaton press (Eaton, 1962) and suspended in two volumes of TNM buffer. The suspension was adjusted to pH 7.4 with 1 M-tris solution, then deoxyribonuclease (2 μg/ml) was added and after incubation for 20 min at 20°C, centrifuged at 30 000 *g* for 30 min. The supernatant (fraction S-30) was centrifuged at 150 000 *g* for 2.5 h and the upper three-fourths of the obtained supernatant were collected (supernatant 150); this was dialysed overnight against the TNM buffer and kept at -40°C. The sedimented ribosomes were used either directly (non-washed ribosomes) or suspended in 1 M-NH_4Cl-tris buffer, left for 24 h with constant stirring at 2°C, then centrifuged at 150 000 *g* for 2.5 h, suspended in TNM buffer and kept at -40°C (washed ribosomes).

Preparation and acylation of tRNA. tRNA was obtained by the method of Zubay (1962). The tRNA to be used for acylation was additionally purified on DEAE-cellulose; for this purpose, to the tRNA solution 2 g of DEAE-cellulose was added per 100 mg of tRNA. After 2 h the cellulose was washed on a filter with 0.2 M-NaCl - 0.01 M-tris-HCl buffer of pH 7.4, then tRNA was eluted with 1 M-NaCl - 0.01 M-tris-HCl buffer of pH 7.4, and precipitated with 2 volumes of cold (-40°C) ethanol, dissolved in water, dialysed against water and stored at -40°C.

Acylation of tRNA was carried out as described by Chomczyński *et al.* (1969).

Incorporation of amino acids. In the experiments, the technique of double labelling with [^{14}C]leucine and [^{3}H]phenylalanine was used. The incorporation of amino acids was carried out under the conditions described previously (Chomczyński *et al.*, 1969). The reference sample contained no poly U. After 40 min of incubation, the reaction was stopped by adding 10% trichloroacetic acid, the precipitated sediment was washed as described by Chomczyński *et al.* (1969) and radioactivity was determined in the Packard 3003 scintillation counter using the technique of double counting of ^{3}H and ^{14}C (De Wachter & Fiers, 1967).

Binding of [^{14}C]*leucyl-tRNA and* [^{14}C]*phenylalanyl-tRNA to the poly U-ribosome complex.* The experiments on the binding of aminoacyl-tRNA's to ribosomes were carried out by the method of Nirenberg & Leder (1964). The incubation mixture (0.05 ml) consisted of 0.1 M-tris-HCl buffer of pH 7.4, 0.07 M-NH_4Cl, varying amounts of magnesium acetate (0.5 - 40 mM), ribosomes (2 E_{260} units), 60 pmoles (5.4 nCi) of [^{14}C]leucyl-tRNA or 21 pmoles (2.5 nCi) of [^{14}C]phenylalanyl-tRNA, and 7 μg of poly U. In each experiment a parallel mixture without poly U was used as a reference sample. After incubation for 20 min at 24°C, the sample was diluted with 3 ml of cooled buffer P containing magnesium acetate at the same concentration as applied in the incubation mixture. Then the sample was filtered through a millipore filter (HAWP 0.45 μ, Millipore Corp., Bedford, Mass., U.S.A.). The sediment on the filter was washed with buffer P (4 times with 5 ml portions), dried, and the radioactivity of the polyU-ribosome-aminoacyl-tRNA complex was determined in a scintillation counter.

RESULTS

The *E. coli* ribosomes exhibiting ambiguity of poly U translation were obtained by washing with 1 M-NH_4Cl-tris buffer. The data presented in Table 1 indicate that the ribosomes washed with the 0.5 M-NH_4Cl-tris buffer did not differ in poly U translation from the non-washed ribosomes. On the other hand, the ribosomes washed with 1 M-NH_4Cl-tris buffer showed a considerable increase in ambiguity of translation. The ambiguity, expressed as percentage of leucine incorporation in comparison to that of phenylalanine incorporation, amounted to 58%. With some preparations, the incorporation of leucine in the presence of poly U was equal to the incorporation of phenylalanine.

When the ribosomes washed with the 1 M-NH_4Cl-tris buffer were kept either in TNM buffer or in 1 M-NH_4Cl-tris buffer at 2°C, an increase of phenylalanine incorporation and a decrease of leucine incorporation were observed during storage

Table 1

The effect of washing of E. coli *ribosomes on the fidelity of poly U translation*

The ribosomes obtained from fraction S-30 (non-washed) were submitted to a procedure of washing for 20 h in tris buffer containing 0.5 M or 1 M-NH_4Cl, then centrifuged for 2.5 h at 150 000 *g* (max.), suspended in TNM buffer and used for experiments on amino acid incorporation. Conditions of the incorporation were as described in Methods.

Preparation of ribosomes	Incorporation of amino acids (nmol/mg of ribosomal protein)		Ambiguity (%)
	[^{3}H]phenylalanine	[^{14}C]leucine	
Non-washed	3.78	0.122	3
Washed in 0.5 M-NH_4Cl-tris buffer	3.98	0.205	4
Washed in 1 M-NH_4Cl-tris buffer	6.12	3.51	58

Table 2

The effect of storage of E. coli *B ribosomes in* 1 M-NH_4Cl-*tris buffer on poly U-dependent incorporation of phenylalanine and leucine*

Ribosomes were kept in 1 M-NH_4Cl-tris buffer at 2°C, and at indicated time intervals samples of 10 µl (3 E_{260} units) were withdrawn and transferred to the incubation mixture for the incorporation of [^{3}H]phenylalanine and [^{14}C]leucine. The composition of the incubation mixture and the conditions of incorporation of amino acids were as described in Methods.

Time of storage of ribosomes	Incorporation of amino acids (nmol/mg of ribosomal protein)		Ambiguity (%)
	[^{3}H]phenylalanine	[^{14}C]leucine	
0.5 h	10.50	4.99	48
1 day	10.53	4.93	47
2 days	10.84	4.85	45
3 days	12.27	2.87	23
4 days	13.21	2.38	18
5 days	15.75	2.64	17
6 days	15.39	1.65	11
7 days	15.22	1.43	9
8 days	15.43	1.48	10
9 days	15.54	1.42	9

(Table 2), indicating that the ribosomes were able to regain the ability of poly U translation in agreement with the genetic code. After two days the ribosomes still retained the ability of ambiguous translation amounting to 50%, which they had gained on washing, but on longer storage the ability to incorporate leucine decreased with a simultaneous increase in phenylalanine incorporation. After six days the ambiguity of translation decreased to about 10% and was maintained at this value for some days. In the control experiment, non-washed ribosomes were stored in TNM buffer and the ambiguity of poly U translation was measured. Under these conditions of storage, the non-washed ribosomes retained high fidelity of poly U translation through all the period studied (six days).

The above experiments not only show the restoration by storage of the correct translation of poly U but also indicate that the ribosomes stored in 1 M-NH_4Cl-tris buffer maintained high activity in incorporation of phenylalanine in the presence of poly U, and could serve as a good preparation for studying the translation of synthetic matrices.

As mRNA recognition is dependent on the codon-anticodon interaction, we have examined the interaction between aminoacyl-tRNA's and the poly U-ribosome complex. For this purpose, experiments were carried out, in the presence of varying concentrations of Mg^{2+}, on the poly U-directed binding of [^{14}C]leucyl-tRNA and [^{14}C]phenylalanyl-tRNA with non-washed ribosomes, with ribosomes washed with 1 M-NH_4Cl-tris buffer, and with ribosomes washed and stored in this buffer (Table 3). At all magnesium concentrations studied, poly U stimulated the binding of phenylalanyl-tRNA to non-washed ribosomes. The binding increased with increasing

concentrations of Mg^{2+}. No binding of leucyl-tRNA was observed under the same conditions. On the other hand, the washed ribosomes in the presence of poly U bound leucyl-tRNA. These results are in agreement with those obtained in experiments on incorporation of phenylalanine and leucine with ribosomes non-washed, and washed with 1 M-NH_4Cl (Table 1). After 7 days of storage of the washed ribosomes, the ability to bind leucyl-tRNA was lower in comparison with the ribosomes examined directly after washing.

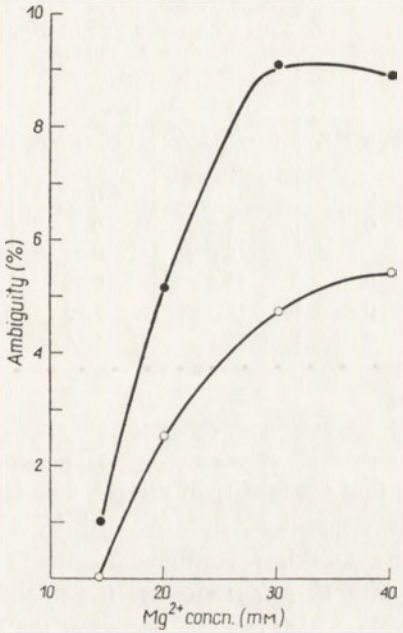

Fig. 1. Ambiguity of binding of leucyl-tRNA and phenylalanyl-tRNA to the poly U-ribosome complex. The binding of [^{14}C]leucyl-tRNA and [^{14}C]phenylalanyl-tRNA to *E. coli* B ribosomes in the presence of poly U was carried out as described in Methods. The ambiguity is defined by the percentage ratio of leucyl-tRNA to phenylalanyl-tRNA bound to ribosomes at different Mg^{2+} concentrations. The ambiguity was determined in the presence of ribosomes: ●, just after washing with 1 M-NH_4Cl-tris buffer, and ○, washed and stored for 7 days in the same buffer.

The interaction of phenylalanyl-tRNA both with ribosomes which had been stored and with those examined directly after washing, did not undergo significant changes. This led to a decrease in the leucyl-tRNA to phenylalanyl-tRNA ratio, which in the presented experiments serves as a measure of the ambiguity of translation in the system studied.

The ambiguity of binding of leucyl-tRNA and phenylalanyl-tRNA to ribosomes at different Mg^{2+} concentrations is presented in Fig. 1. The ambiguity, measured by the codon-anticodon interaction, was dependent on the Mg^{2+} concentration and was twice as great in the systems containing the ribosomes examined directly after washing than with the ribosomes stored for 7 days.

Table 3

Binding of [^{14}C]*phenylalanyl-tRNA and* [^{14}C]*leucyl-tRNA to the poly U-ribosome complex*

In the experiments were used: non-washed ribosomes; ribosomes washed with 1 M-NH_4Cl-tris buffer; and ribosomes stored in this buffer at 2°C for 7 days. The results for [^{14}C]phenylalanyl--tRNA and [^{14}C]leucyl-tRNA represent the net values obtained after subtracting the amount of aminoacyl-tRNA's bound to ribosomes in the absence of poly U. The conditions of incubation were as described in Methods.

Mg^{2+} concn. (mM)	Binding of aminoacyl-tRNA's (pmol/2 E_{260} units of ribosomes)					
	Non-washed ribosomes		Washed ribosomes		Ribosomes stored for 7 days	
	Phe-tRNA	Leu-tRNA	Phe-tRNA	Leu-tRNA	Phe-tRNA	Leu-tRNA
5	5.2	0	5.2	0	4.4	0
10	8.1	0	7.9	0	7.4	0
14	—	—	8.0	0.06	7.7	0
20	9.3	0	12.8	0.65	12.5	0.34
30	10.1	0	13.2	1.20	12.9	0.62
40	10.6	0	13.3	1.14	13.1	0.74

DISCUSSION

It has been postulated that the fidelity of mRNA translation is dependent on the ribosome structure. This suggestion, put forward by Davies, Gilbert & Gorini (1964), was confirmed by Ozaki, Mizushima & Nomura (1969). These authors have demonstrated that with *E. coli* ribosomes the fidelity of poly U translation is dependent on P-10 protein, which is a component of the 30-S subunit of the ribosome. Studies on the fidelity of translation carried out with the use of aminoglycoside antibiotics, such as streptomycin (Ozaki *et al.*, 1969), spectinomycin (Anderson, Davies & Davis, 1967; Bollen, Davies, Ozaki & Mizushima, 1969), kasugomycin (Tanaka, Yoshida, Sashikata, Yamaguchi & Umezawa, 1966; Sparling, 1970) indicate that protein P-10 is not the only component of the ribosome responsible for the fidelity of translation. The experiments carried out in this laboratory (Chomczyński *et al.*, 1969; Perzyński *et al.*, 1969) also showed that fidelity of translation is dependent on ribosome structure. It has been demonstrated that *B. stearothermophilus* ribosomes contain substance(s) indispensable for accurate recognition of poly U. Removal of these substances by washing the ribosomes in 0.5 M-NH_4Cl, dissociation of ribosomes and washing in 0.2 M-sucrose led to an increase in the ambiguity of poly U translation. Addition of the wash to the incorporation system containing the washed ribosomes, restored to a considerable extent the fidelity of translation. As we have applied a mild procedure for washing out these substances from *B. stearothermophilus* ribosomes, they are different from the P-10 protein which became dissociated from the ribosomes in a solution of 4 M-LiCl - - 8 M-urea. It has also been demonstrated by Szafrański *et al.* (1970) that the *E. coli*

ribosomes are more resistant to factors leading to ambiguity of translation than the *B. stearothermophilus* ribosomes, and that these differences are most probably due to differences in the structure of ribosomes from these two sources.

The evidence obtained in the present work indicates that washing of *E. coli* ribosomes with a solution containing 1 M-NH_4Cl led to a high ambiguity of poly U translation. This ambiguity, expressed as the percentage of leucine incorporated in comparison with the amount of incorporated phenylalanine, was about 50% and was equal to the ambiguity obtained in the system with *B. stearothermophilus.*

The ambiguity of translation with the washed *E. coli* ribosomes disappeared during storage of the ribosomes. This phenomenon was confirmed by means of the interaction of [^{14}C]phenylalanyl-tRNA and [^{14}C]leucyl-tRNA with the poly U-ribosome complex. The *E. coli* ribosomes washed with 1 M-NH_4Cl gained the ability to bind leucyl-tRNA in the presence of poly U which indicates their influence on the incorrect codon-anticodon interaction; this incorrect interaction decreased during storage of the ribosomes.

From a comparison of the ambiguity of translation measured by binding of aminoacyl-tRNA's to the poly U-ribosome complex, with the ambiguity measured by incorporation of phenylalanine and leucine it may be seen that the number of mistakes is greater in the latter case. This is probably due to the fact that the conditions of binding of aminoacyl-tRNA's to the poly U-ribosome complex do not fully correspond to the processes occurring during protein biosynthesis. However, the changes in the ambiguity of translation observed in both types of experiments showed the same tendencies indicating that the incorrect translation of poly U is related to the codon-anticodon interaction. The ambiguity of poly U translation could be caused by the incorrect recognition of the UUU codon by the leucyl-tRNA population with the CAA anticodon. Such a possibility has been suggested by Kan, Nirenberg & Sueoka (1970).

An interesting hypothesis has been published by Kurland (1970) who suggests that the structure of ribosomes and codon of mRNA are involved in the formation of the stereospecific barrier which prevents ambiguous binding of tRNA to the mRNA-ribosome complex. The present experiments are in agreement with this hypothesis and indicate that the incorrect interaction between the codon and anticodon could be facilitated by structural changes in *Escherichia coli* B ribosomes evoked by washing with solutions of high salt concentration.

This work was supported in part by Grant no FG-Po-232 from the U.S. Department of Agriculture.

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WPŁYW WYSOKICH STĘŻEŃ SOLI NA WIERNOŚĆ TRANSLACJI Z RYBOSOMAMI *ESCHERICHIA COLI*

Streszczenie

1. Wykazano, że płukanie rybosomów *E. coli* B przy wysokim stężeniu soli buforem tris z 1 M-NH_4Cl prowadzi do znacznego wzrostu dwuznaczności translacji poli U w układzie z tymi rybosomami.

2. Kilkudniowe przechowywanie płukanych rybosomów *E. coli* B w tym samym buforze w temp. 2°C powoduje spadek dwuznaczności translacji poli U.

3. Powstawanie i zanik dwuznacznego odczytywania poli U z rybosomami płukanymi i przechowywanymi stwierdzono również za pomocą wiązania aminoacylo-tRNA do kompleksu poli U - - rybosomy.

Received 24 November, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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METABOLISM OF GLUCOSE IN THE SUBLINGUAL GLAND OF THE RAT, AND THE EFFECT OF INSULIN *IN VITRO*

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The metabolism of glucose in rat sublingual gland was studied using [U-^{14}C]glucose. Slices of the glands were found to incorporate the radioactivity into mucoprotein, oligosaccharides, lactate, citrate, malate, free amino acids and CO_2. The addition of insulin resulted in a twofold increase in the uptake of glucose from the medium, a threefold increase in mucoprotein synthesis as well as in enhanced synthesis of amino acids, but it had no effect on the incorporation of the label into other metabolites of glucose.

Salivary glands are the site of biochemical processes leading to formation of saliva, a mucoprotein-rich fluid. The activity of salivary glands is controlled by several hormones, including thyroxine, adrenocorticosteroids and testosterone (Baker, Clapp & Light, 1964; Liu & Lin, 1969). So far, the effect of insulin on the synthesis of particular components of salivary glands and saliva has not been studied. However, both in patients with diabetes and in alloxan-diabetic rats, swelling of salivary glands, decrease in secretion of saliva, and increased incidence of dental caries have been observed; this seems to indicate that deficiency of insulin affects the metabolism of salivary glands.

In an earlier work concerning the overall metabolism of [^{14}C]glucose in submaxillary gland of the rat *in vitro*, Masi, Pocchiari & Szymczyk (1966) demonstrated that insulin stimulated the incorporation of the label into sialic acid and hexosamine present in mucoprotein of the gland, and had no effect on other metabolites of glucose or on consumption of oxygen.

In this paper we present the experiments on the effect of insulin on the metabolism of glucose in the sublingual gland *in vitro*.

MATERIALS AND METHODS

Animals. Male Wistar rats 8 - 10 weeks old, weighing 150 - 200 g, were used. The animals had free access to water and were fed *ad libitum* the standard LSM mixture (Bacutil, Warszawa, Poland) supplemented with carrots. The mixture

contained: bran, ground grain, fish meal, skimmed-milk powder, yeast, mineral salts and vitamins.

Isolation of salivary glands. The animals were fasted for 18 h, stunned by a strong blow on the back of the neck, the spine at the neck cut through and, after bleeding, the capsulae containing the submaxillary and sublingual glands were isolated. The glands were removed from the capsula, rinsed with cold 0.9% NaCl solution and blotted. Separation of two small glands lying in a common connective-tissue capsula presents some difficulties, therefore the outline of the two glands was made visible by pressing them between watch-glasses; then the glands were separated. In preliminary experiments the correct separation of the glands was checked histochemically taking into account that in the sublingual gland the mucine components are predominant. The histochemical staining was made with haematoxylin and eosine (basic staining), with alcian blue for acidic mucopolysaccharides and with PAS for neutral mucopolysaccharides.

Conditions of incubation. Slices of the sublingual gland, about 0.5 mm thick, were prepared immediately after isolation of the gland, with a microtome constructed as described by Stadie & Riggs (1944). The slice weighing about 50 mg was placed in a Warburg flask (kept in ice) containing 1.2 ml of Krebs phosphate buffer, pH 7.4 (Krebs & Henseleit, 1932) and 1.2 mg of non-radioactive and [U-^{14}C] glucose (total radioactivity 20 μCi). In the centre well was placed a strip of Whatman no. 1 paper which carried 0.3 ml of 30% NaOH solution. The incubation was for 60 min in an atmosphere of pure oxygen, at 37°, and the consumption of oxygen was measured. The incubation was stopped by placing the flasks in crushed ice. In the experiments on the effect of insulin, this hormone was added to a concentration of 0.1 unit/ml of the incubation medium.

Procedure applied after incubation. The radioactive metabolites were determined in: *a*, incubation medium; *b*, tissue extract made with 60% ethanol; *c*, the residue after alcoholic extraction; and *d*, the evolved CO_2.

a. The incubation medium was collected and stored at -20° before being used for chromatography.

b. The tissue slice was washed three times with physiological saline solution, then homogenized with 2 ml of cold 60% ethanol, left for at least 2 h at 4°C, centrifuged at 5000 *g*, and the obtained sediment washed once with 1 ml of 60% ethanol and twice with 1-ml portions of water. The supernatant and the washes were pooled and evaporated at 20 - 25° in a Unipan (Warszawa, Poland) evaporator, and the dry residue was dissolved in 1 ml of water and kept at -20°.

c. The washed tissue residue remaining after the ethanol extraction was treated with 5 ml of an ethanol - acetone mixture to extract lipid fractions, and the radioactivity of the lipid fraction and of protein was measured.

d. To determine the radioactivity of the CO_2 absorbed by the NaOH-carrying paper, the paper was transferred to a tube containing CO_2-free water, and the central well washed three times with water. CO_2 was precipitated by adding 1 ml of 10% $BaCl_2$ solution which contained 0.5 ml of non-radioactive 1.2% Na_2CO_3. The

sediment was washed three times with water, suspended in a small volume of water and quantitatively transferred to an aluminium planchette. After drying under an infrared lamp, the radioactivity was measured. The weight of the sediment served for calculation of the absorption coefficient.

Chromatography. Chromatographic separation of the incubation medium and the tissue extract was carried out on Whatman no. 1 paper using the following solvent systems: 1, *tert*.-butanol - picric acid - water (80:2:20, v/w/v); 2, butan--1-ol - acetic acid - water (40:11:25, by vol.); 3, butan-2-ol - 85% formic acid - water (75:15:10, by vol.); 4, phenol - water - aq. ammonia (sp. grav. 0.91), (80:20:1, w/v/v).

Descending chromatography with solvent system 1 was used for separation of glucose and oligo- and polysaccharides (Fig. 1); maltotriose and maltotetrose were identified by their R_F values and susceptibility to amylase. The amount of the material applied on the paper was 10 - 20 µl of the incubation medium and 100 µl of the tissue extract preparation. Lactic acid was determined in 20-µl samples by the descending technique with solvent system 2 (Fig. 2). To fix the lactic acid, the developed chromatogram was placed in an atmosphere of ammonia.

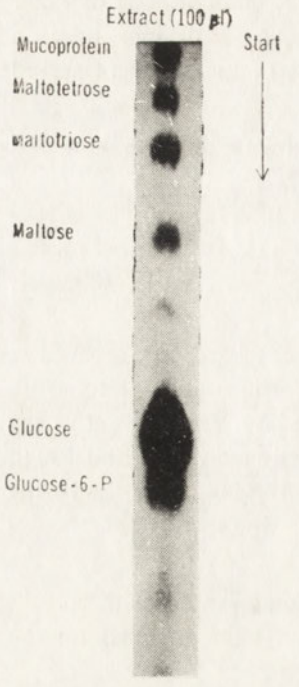
Amino acids were separated by two-dimensional chromatography with solvent systems 3 and 4 (Fig. 3), 100 µl of the material studied being applied on the paper.

Radioactivity estimations. Radioactivity on the chromatograms was detected by radioautography with Super-R X-ray film (Foton, Warszawa, Poland), which was exposed for about 10 days with application of intensifying screens (Perlux, Veb Kali-Chemie, Berlin). Radioactivity of the identified spots was determined with an automatic chromatogram scanner (Frieseke & Hoepfner, GmbH, Erlangen--Bruck, West Germany), the paper background being subtracted. Radioactivity in samples was determined in an end-window Geiger-Müller BOH-42 counter (P.I.E., Warszawa, Poland).

Calculation. The radioactivity determined in the metabolites (found in the material separated as described in *Procedures applied after incubation*) was taken as a measure of the metabolized glucose, which was expressed as µg/50 mg of tissue/1 h.

The significance of the differences between the means for the samples with and without insulin was determined by Student's *t* test.

Special reagents. [U-^{14}C]Glucose (Radiochemical Centre, Amersham, England, or UVWR, Prague, Czechoslovakia); ^{14}C-labelled amino acids used as standards and labelled citric and malic acids (Radiochemical Centre, Amersham). Crystalline insulin, 25 u./mg (Tarchomińskie Zakłady Farmaceutyczne Polfa, Warszawa, Poland). Solvents for chromatography: butan-1-ol (Zakłady Chemiczne Estron, Warszawa, Poland); butan-2-ol and *tert*.-butanol (Reanal, Budapest, Hungary); acetic acid and phenol (Zakłady Chemiczne Oświęcim, Poland); 85% formic acid (Polskie Odczynniki Chemiczne, Gliwice, Poland); picric acid (Wytwórnia Chemiczna, Krupski Młyn, Poland); ammonia sp. grav. 0.91 (Zakłady Azotowe, Tarnów, Poland).



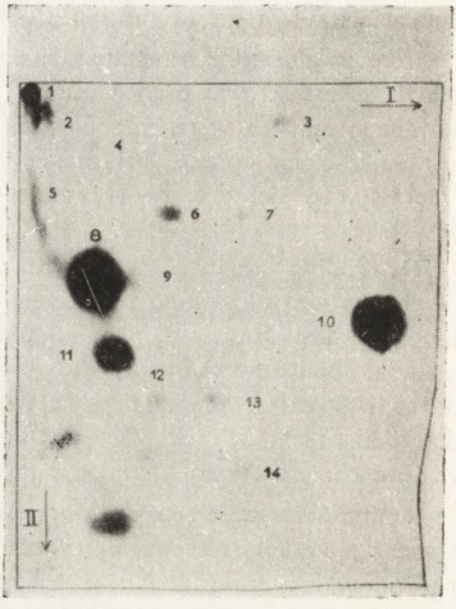


Fig. 1 Fig. 2 Fig. 3

Fig. 1. Autoradiochromatogram of the water-ethanol extract from sublingual gland after incubation with [U-^{14}C]glucose. The chromatogram was developed in solvent system 1. The non-identified spots of R_F values close to that for glucose were also present in the [U-^{14}C]glucose of Czechoslovakian production whereas the labelled glucose from the Amersham Centre was chromatographically pure.

Fig. 2. Autoradiochromatogram of the water-ethanol extract from sublingual gland after incubation with [U-^{14}C]glucose. The chromatogram was developed in solvent system 2.

Fig. 3. Autoradiochromatogram of the water-ethanol extract from sublingual gland after incubation with [U-^{14}C]glucose. Two-dimensional chromatogram, developed with solvent system 3 in the first dimension (I) and with solvent system 4 in the second (II). 1, Mucoprotein; 2, glucose-6-phosphate; 3, citrate; 4, aspartate; 5, maltotriose; 6, glutamate; 7, malate; 8, glucose; 9, serine; 10, lactate; 11, glycine; 12, glutamine; 13, alanine; 14, γ-aminobutyrate.

RESULTS

Rat sublingual gland exhibited a high degree of endogenous respiration which did not increase on addition of glucose over the concentration range of 0.05 - 5%. The consumption of oxygen during 1-h incubation by a 50-mg slice was on average 43 µl which corresponds to 1.92 µmoles. The addition of insulin had no effect on the consumption of oxygen. Chromatographic analysis of the products formed on incubation of the slice of sublingual gland with [U-^{14}C]glucose showed incorporation of the label into carbon dioxide, mucoprotein, oligosaccharides (maltose, maltotriose and maltotetrose), lactate, amino acids (aspartate, glutamate, γ-amino-

butyrate, glutamine, serine, glycine and alanine) and in trace amounts, into citrate, malate, glycogen and some other, non-identified compounds (Fig. 3). No radioactivity was found in the lipid fraction. The addition of insulin to the incubation medium had no effect on the qualitative pattern of glucose metabolites.

Table 1

Metabolism of glucose in slices of rat sublingual gland and the effect of insulin

The tissue slice, 50 mg, was incubated for 1 h at 37° in an atmosphere of O_2, in 1.2 ml of phosphate buffer, pH 7.4, containing 0.1% of non-radioactive and [U-^{14}C]glucose (total radioactivity 20 μCi). Insulin was added to a concentration of 0.1 u./ml. For details see Methods. Mean values from 6 experiments, ±S.E., are given. The results are expressed as μg of glucose per 50 mg of tissue per 1 h.

	Without insulin	With insulin	*t*	Significance
Glucose uptake	197 ±11.02	335 ±19.9	6.07	+
Glucose recovered in:				
Mucoprotein	32.5± 2.31	103.2± 4.5	13.97	+
Oligosaccharides	2.0± 0.257	2.8± 0.353	1.86	—
Lactate	70.7± 7.82	80.0± 3.56	1.08	—
Amino acids	16.5± 3.662	30.0± 3.41	2.70	+
CO_2	35.1± 2.54	42.0± 2.97	1.76	—
Glucose with G-6-P in tissue extract	18.6± 2.91	25.0± 3.46	1.41	—
Total recovered	175.4 (89%)	283.0 (84.5%)		

The quantitative determinations of glucose metabolism are shown in Table 1. The uptake of [^{14}C]glucose from the incubation medium was on the average 197 μg. Of the radioactivity recovered, 18% was found in mucoprotein, 1.2% in oligosaccharides, 9% in amino acids, 40% in lactate, 20% in CO_2 and about 11% of the radioactivity was found in the glucose and glucose-6-phosphate present in the tissue ethanolic extract. The non-accounted for 11% of glucose radioactivity might correspond to citrate and malate (the radioactivity of which was too low for quantitative determinations) and the three non-identified spots (Fig. 3).

Insulin added to the incubation medium (0.1 u./ml) increased the disappearance of glucose from the medium by about 70% (Table 1). Insulin stimulated threefold the biosynthesis of mucoprotein, and twofold the formation of free amino acids, but had no effect on the amount of glucose remaining in the tissue, or on production of CO_2, lactate and oligosaccharides.

DISCUSSION

The metabolism of [^{14}C]glucose in the sublingual gland was studied by the same methods as that in the submaxillary gland (Masi *et al.*, 1966); a comparison of the obtained results indicates that both these glands have a similar capacity to

metabolize the glucose added to the incubation medium. Also the metabolites formed were similar but formation of glutamine and γ-aminobutyrate was demonstrated only in the sublingual gland. The radioactivity found in lactate, citrate and malate indicates that in the sublingual gland the glycolytic pathway of glucose metabolism is operative, with subsequent oxidation *via* the citric acid cycle. Formation of radioactive mucoprotein, demonstrated previously in the submaxillary gland (Masi *et al.*, 1966) and presently in the sublingual gland, suggests that both these glands possess the mechanism required for biosynthesis of the carbohydrate moiety of mucoprotein.

Insulin added to the incubation medium had little effect on the metabolism of glucose in the submaxillary gland (Masi *et al.*, 1966). On the other hand, in the sublingual gland insulin enhanced the metabolism to a marked extent.

From the presented results it appears that the sublingual gland of the rat is susceptible to insulin, and it may be suggested that this hormone is involved in the regulation of biosynthesis of salivary mucoprotein.

The authors wish to express their gratitude to Prof. Dr. S. Barański of the Military Institute of Aviation Medicine, Warszawa, for making available the automatic chromatogram scanner. Thanks are also due to the Tarchomin Pharmaceutical Plant "Polfa", Warszawa, for the kind gift of insulin.

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METABOLIZM GLUKOZY W GRUCZOLE PODJĘZYKOWYM SZCZURA I WPŁYW INSULINY *IN VITRO*

Streszczenie

Metabolizm glukozy w gruczole podjęzykowym szczura badano przy użyciu [U-^{14}C]glukozy. Stwierdzono, że skrawki gruczołu włączają radioaktywność w mukoproteid, oligosacharydy, mleczan, cytrynian, jabłczan, wolne aminokwasy i CO_2. Dodanie insuliny zwiększało prawie dwukrotnie pobieranie glukozy ze środowiska, zwiększało trzykrotnie syntezę mukoproteidu, oraz syntezę aminokwasów, ale nie miało wpływu na włączanie radioaktywności do innych metabolitów glukozy.

Received 26 November, 1970

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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EFFECT OF ALLOXAN-DIABETES ON THE CONTENT OF SIALIC ACIDS AND ACTIVITY OF URIDYLYLTRANSFERASES IN RAT SALIVARY GLANDS

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1. In rat sublingual and submaxillary gland, sialic acids were found only in the bound form. The activity of uridylyltransferases which catalyse the formation of UDPglucose, UDPgalactose and UDPacetamidoglucose was demonstrated in the extracts of both glands. 2. In alloxan-diabetic rats a marked decrease in the weight of both glands was observed, associated with a decrease in the content of protein and sialic acids. The activity of the enzymes studied was decreased by about 50% in the submaxillary gland and to a much greater extent in the sublingual gland.

It has been demonstrated that in rat submaxillary gland (Masi, Pocchiari & Szymczyk, 1966) and sublingual gland (Szymczyk & Jachimowicz, 1971) insulin stimulates *in vitro* the incorporation of the ^{14}C-label of glucose into carbohydrate moiety of mucoprotein. The present work describes the effect of alloxan-diabetes on the protein and sialic acid content in rat salivary glands. Moreover, four reactions involved in biosynthesis of hexosamines and sialic acids from glucose were examined, namely the formation of UDPglucose, UDPgalactose, UDPaminoglucose and UDPacetamidoglucose.

MATERIALS AND METHODS

Animals. Male Wistar rats 8 - 10 weeks old, weighing 150 - 200 g, were used. The conditions of feeding of both control and alloxan-treated rats, their killing, and isolation of the salivary glands were as described by Szymczyk & Jachimowicz (1971).

Alloxan-diabetes was induced as described by Klebanoff & Greenbaum (1954) by a single intraperitoneal injection of alloxan monohydrate (160 mg/kg body weight) o 18-h fasted rats. Alloxan was administered as a 5% solution in 0.125 M-phosphate-citrate buffer, pH 4. The blood sugar was tested by the method of Hagedorn & Jensen (1923), and as early as two days after administration of alloxan it was found to range from 200 to 900 mg%. To increase the survival, which under our

conditions amounted to 70%, 2 ml of 40% aqueous solution of glucose was given for three days by stomach intubation both to the alloxan-diabetic and control rats.

The loss of body weight 8 days after alloxan administration amounted to about 20% whereas the control animals during the same period showed an about 13% gain in weight (Table 1). The loss of body weight was so significant that, together with hyperglycaemia, it was accepted as the characteristic feature of the diabetes. The results for the alloxan-treated animals in which the blood sugar was higher than 200 mg% but which showed no loss in weight, were found to be similar to those of the control group and were not included in the calculations of the average values. Such animals constituted about 5% of the total number studied.

Oxygen consumption was determined by the Warburg manometric technique as described by Szymczyk & Jachimowicz (1971).

Determination of sialic acids. The submaxillary and sublingual glands were homogenized each in 3 ml of 0.05% sulphuric acid in an all-glass Potter-type homogenizer kept on ice. A part of the homogenate, in which free sialic acids were to be determined, was left for 60 min at 4°C; another part, to be used for determination of total sialic acids, was heated for 60 min at 80°C. After centrifugation for 30 min at 5000 rev./min, the volume of the supernatants was measured and sialic acids were determined in duplicate samples by the colorimetric method of Warren (1959). To correct for the interference by deoxyribose, the amount of sialic acids (as *N*-acetylneuraminic acid) was calculated from the values: $0.09\ E_{549\ nm} - 0.033\ E_{532\ nm}$ (Warren, 1959).

Determination of enzymic activities. The isolated salivary glands were homogenized in 19 vol. of 0.9% NaCl solution in an all-glass Potter-type homogenizer and centrifuged at 70 000 *g* for 3 h at 4°C. The obtained supernatant was used for determination of activity of uridylyltransferases.

The activity of UTP : α-D-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) was determined in an incubation mixture containing in a final volume of 0.4 ml: 10 μmoles of tris-HCl buffer, pH 7.0, 2 μmoles of magnesium acetate, 0.1 μmole of glucose-1-[^{32}P]phosphate, 0.2 μmole of UTP, and supernatant (60 - 70 μg protein). The incubation time was 10 min.

The activity of UDPglucose : α-D-galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) was assayed in an incubation mixture containing in a final volume of 0.4 ml: 20 μmoles of glycine buffer, pH 8.1, 100 μmoles of EDTA, 0.1 μmole of galactose-1-[^{32}P]phosphate, 0.1 μmole of UDPglucose, and supernatant (80 - - 100 μg of protein). The incubation time was 30 min.

The activity of UTP : 2-amino-2-deoxy-α-D-glucose-1-phosphate uridylyltransferase and UTP : 2-acetamido-2-deoxy-α-D-glucose-1-phosphate uridylyltransferase (EC 2.7.7.23) was measured in an incubation mixture which contained in a final volume of 0.4 ml : 10 μmoles of tris-HCl buffer, pH 7.0, 2 μmoles of magnesium acetate, 0.2 μmole of glucose-1,6-diphosphate, 0.1 μmole of UTP, 0.05 μmole of aminoglucose-6-[^{32}P]phosphate (or 0.05 μmole of *N*-acetamidoglucose-6-[^{32}P]phosphate), and supernatant (100 μg of protein). The incubation time was 30 min.

In all the assays, the enzymic activity was linear with the indicated incubation time, and with the amount of the supernatant protein.

The assays were made on duplicate samples; the reactions were carried out at 37°C and stopped by adding 0.4 ml of 10% trichloroacetic acid (cooled to 0°C). The samples were left on ice for 10 min, then centrifuged, and to the supernatants 50 mg of charcoal suspended in 1 ml of water was added to absorb the nucleotides. After 30 min the charcoal was centrifuged, washed three times with water cooled to 0°C, transferred to a planchette, and the radioactivity was determined in a Geiger--Müller end-window BOH-42 counter (P.I.E., Warszawa, Poland).

The activity of the enzymes was calculated from the radioactivity of the utilized substrate incorporated into diphosphonucleosidesugars adsorbed on active charcoal.

For identification of the formed ^{32}P-labelled compounds, the nucleotides were eluted from the charcoal, three times with 2-ml portions of 30% ethanol containing 0.1% of NH_4OH. The eluates were concentrated at 30°C, and chromatographed by the ascending technique on Whatman no. 1 paper in the solvent system of Ebel (1952) consisting of isopropanol - 20% trichloroacetic acid - 25% NH_3 (75:25:0.3, by vol.). For autoradiography Super-R X-ray plate (Foton, Warszawa, Poland) was used. The exposition time was 10 days.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as standard. For determination of total protein in the salivary glands, the homogenate was treated with an equal volume of 10% trichloroacetic acid, centrifuged, and the precipitate dissolved in 1 N-NaOH.

Phosphorus was determined by the method of Fiske-Subbarow in the modification of Lohmann & Jendrassik (1926).

Reagents. The radioactive substrates for enzymic assays were prepared in the following way. [^{32}P]Glucose-1-phosphate was obtained enzymically as described by McCready & Hassid (1944) using potato extract for starch phosphorolysis in the presence of [^{32}P]orthophosphate. The radioactivity of the preparations ranged from 2×10^5 to 4×10^5 counts/min/µmol. [^{32}P]Galactose-1-phosphate was prepared from galactose, using yeast (*Saccharomyces fragilis*) galactokinase, in the presence of [^{32}P]ATP, according to the method described by Trucco, Caputto, Leloir & Mittelman (1948). Specific activity of the preparations was 10^5 counts/min/µmol. [^{32}P]-Aminoglucose-6-phosphate was obtained from aminoglucose and [^{32}P]ATP according to the method of Preiss & Greenberg (1967). The specific activity of the preparation was 8×10^5 counts/min/µmol. [^{32}P]Acetamidoglucose-6-phosphate was prepared by acetylation of [^{32}P]aminoglucose-6-phosphate with acetic anhydride. Specific activity of the preparations was 2×10^5 to 3×10^5 counts/min/µmol. The purity of the synthesized labelled compounds was checked by paper chromatography and by cochromatography with standard compounds.

Other reagents were from the following sources: galactose (Pfanstichl Chem. Co., Wankegan, Ill., U.S.A.); glucose (F. O. Ch., Gliwice, Poland); aminoglucose (Light, Colnbrook, Bucks., England); glucose-1-P, glucose-6-P, galactose-1-P and alloxan monohydrate (Fluka A. G., Buchs S. G., Switzerland); sodium salts of ATP, GTP, ITP, CTP and UTP (Sigma Chem. Comp., St. Louis, Mo., U.S.A.);

the concentration of the dissolved nucleotides was estimated spectrophotometrically and calculated from the molar extinction coefficient given by the manufacturers; UDPglucose (Boehringer Soehne, GmbH, Mannheim, West Germany); crystalline bovine serum albumin (Pentox Incorp., Kankakee, Ill., U.S.A.); *N*-acetylneuraminic acid (Serva, Heidelberg, West Germany); tris (Koch-Light Lab., Colnbrook, England); thiobarbituric acid (British Drug Houses, Poole, Dorset, England); [^{32}P]-H_3PO_4 (Institute for Nuclear Research, Warszawa, Poland); [γ-^{32}P]ATP was prepared and kindly given by Doc. Dr. T. Chojnacki; charcoal (Zakłady Elektrod Węglowych, Racibórz, Poland) was prepared as described by Threlfall (1957); sodium periodate (meta) (T. Schuchardt, Munchen, West Germany); sodium (meta) arsanite (W.O.Ch., Złoty Stok, Poland); cyclohexanone (Tarnowskie Zakłady Azotowe, Tarnów, Poland).

RESULTS

Protein and sialic acid content and weight of salivary glands. In the alloxan--diabetic rats, together with the decrease in body weight, there was a decrease in the weight of the salivary glands (Table 1). The decrease was observed on the fifth day after administration of alloxan and was greater in the sublingual than in the submaxillary gland; the amount of total protein in the glands diminished parallelly (Table 2). However, when calculated per gram of tissue, the content of protein appeared to be unaltered.

Sialic acids were found to be present inthe glands in the bound form only (Table 3). Their content amounted in normal rats to about 0.66 μmole per one submaxillary gland, whereas in the sublingual gland it was twice as high, and even seven times as high when calculated on the weight basis. After alloxan administration, changes in sialic acids were statistically significant in the sublingual gland beginning with the third day and in the submaxillary gland, beginning with the fifth day; on the

Table 1

The effect of alloxan-diabetes on body weight of the animals and weight of salivary glands

The figures represent mean values, and for the weight of the glands, mean values ±S.E. For the diabetic animals, days since the injection of alloxan are indicated.

Rats	No. of animals	Glycaemia (mg%)	Change in body weight (%)	Weight of pair of glands	
				submaxillary (mg)	sublingual (mg)
Control	16	106	+13	250 ±12.4	64 ± 2.5
Diabetic					
3 day	4	490	— 7	247 ±39.5	72 ±24.1
5 day	6	517	—20	177*± 5.1	36*± 3.5
8 day	9	340	—17	150*± 9.6	23*± 3.2

* Statistically significant difference; $t = 2.1$, $P < 0.05$.

Table 2

The effect of alloxan-diabetes on the content of total protein in salivary glands of the rat

The conditions of the experiments were as described in Methods and the numbers of animals as in Table 1. Mean values ±S.E. are given.

Gland	Total protein					
	g/100 g of tissue	% of control	Signi-ficance	mg/pair of glands	% of control	Signi-ficance
Submaxillary						
Control	11.9±0.42	100		27.8±1.52	100	
Diabetic						
3 day	15.5±1.00	130	—	38.6±7.62	140	—
5 day	9.9±0.44	83	—	19.4±3.80	70	+
8 day	11.5±0.84	97	—	16.4±1.26	59	+
Sublingual						
Control	11.3±0.38	100		7.3±0.32	100	
Diabetic						
3 day	15.8±1.77	140	—	7.8±2.20	100	—
5 day	13.1±0.58	115	—	3.9±0.17	53	+
8 day	11.2±0.59	100	—	2.7±0.56	34	+

Table 3

The effect of alloxan-diabetes on the content of bound sialic acids in salivary glands of the rat

The conditions of the experiments were as described in Methods and the numbers of animals as in Table 1. Mean values ±S.E. are given.

Gland	Sialic acids					
	g/100 g of tissue	% of control	Signi-ficance	mg/pair of glands	% of control	Signi-ficance
Submaxillary						
Control	273± 5.64	100		0.66±0.01	100	
Diabetic						
3 day	289±30.4	106	—	0.53±0.04	80	—
5 day	226± 9.82	82	+	0.39±0.01	59	+
8 day	156± 6.5	51	+	0.24±0.01	36	+
Sublingual						
Control	1880±57.3	100		1.19±0.05	100	
Diabetic						
3 day	1566±45.0	83	+	0.85±0.05	71	+
5 day	1021±59.0	54	+	0.38±0.04	32	+
8 day	902±46.5	48	+	0.21±0.02	18	+

eighth day, the decrease was still greater and was more marked in the sublingual gland.

Oxygen consumption by a 50-mg slice of the submaxillary gland amounted on average to 76 µl per hour, and by a corresponding slice of the sublingual gland, to 44 µl. Administration of alloxan to rats had no effect on the consumption of oxygen (Table 4).

Activity of uridylyltransferases. In both glands, the amount of protein in the 70 000 *g* supernatant used for determination of enzymic activities was much lower, when calculated per one pair of glands, in alloxan-diabetic rats than in normal animals. As simultaneously there was a decrease in the weight of salivary glands, the content of protein calculated per 1 g of tissue was practically unaltered (Table 5).

Table 4

Consumption of oxygen by salivary glands of normal and alloxan-diabetic rats

For details see Methods. The diabetic rats were examined 8 days after alloxan administration. The results are mean values from 10 experiments ±S.E.

Rats	O_2 consumption (µl/50 mg/h)	
	Sublingual gland	Submaxillary gland
Control	43.8±1.16	76.1±2.04
Alloxan-diabetic	45.0±2.4	72.6±4.49

Table 5

The amount of protein and the activity of uridylyltransferases in salivary glands of normal and alloxan-diabetic rats

Eight days after alloxan administration, the amount of protein and enzymic activities were determined in the 70 000 *g* supernatant of the tissue homogenate. Activity of the enzymes is expressed as nmoles of the UDPsugar formed. Mean values ±S.E. are given. The number of experiments is given in parentheses; for each experiment three rats were used.

	Rats (expts)	Sublingual gland		Submaxillary gland	
		mg/g tissue	mg/pair of glands	mg/g tissue	mg/pair of glands
Protein in 70 000 *g* supernatant...	normal (19)	41	2.6	72	18
	diabetic (19)	44	1.0	78	12
Uridylyltransferase		UDP-sugar formed, nmoles per minute and			
		per mg protein	per g tissue	per mg protein	per g tissue
UTP : Glucose-1-P	normal (10)	89 ±14.5	3950 ±155	5.5±1.6	447±153
	diabetic (10)	36 ± 5.9	1384 ±171	3.2±0.5	230± 29.5
UDPglucose : galactose-1-P	normal (4)	0.62± 0.1	26 ± 2.3	0.5±0.04	36± 0.9
	diabetic (4)	0.02± 0.003	1.0± 0.2	0.2±0.08	14± 4.8
UTP : *N*-acetamido-glucose-6-P	normal (5)	0.7 ± 0.09	25.0± 3.3	0.2±0.02	13± 0.9
	diabetic (5)	0.3 ± 0.09	12.0± 2.9	0.1±0.001	9± 0.9

Out of the four enzymes studied, the activity of three uridylyltransferases was demonstrated in the supernatants of both salivary glands. The activity, expressed in nmoles of UDPsugar formed per minute per gram of tissues, and per gland, are presented in Table 5. A typical autoradiochromatogram obtained after incubation of one of the enzymes studied, is shown in Fig. 1.

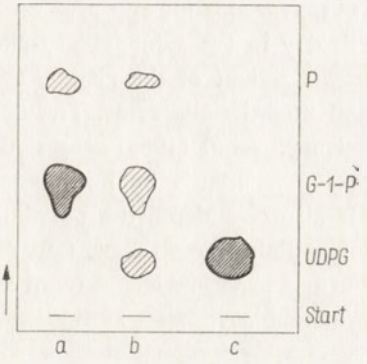

Fig. 1. Autoradiogram of paper chromatograms of: *a*, [^{32}P]glucose-1-phosphate used as substrate; *b*, ^{32}P-labelled substances after incubation; *c*, the formed UDPglucose, adsorbed on charcoal.

In both salivary glands, the enzyme catalysing formation of UDPglucose had the highest activity; however, the activity in the sublingual gland was 8 times as high as in the submaxillary one. UTP was essential for the reaction to occur, and it could not be replaced by either ATP, ITP, GTP or CTP.

The synthesis of UDPgalactose from UDPglucose and galactose-1-P was also demonstrated in both glands. The enzyme activity was the same in both glands, and much lower than that of the former enzyme, 1/20 for the submaxillary gland, and 1/200 for the sublingual one.

The activity of uridylyltransferase UTP : *N*-acetamidoglucose-1-P was assayed with *N*-acetamidoglucose-6-P as substrate. The demonstrated formation of UDP-acetamidoglucose indicates that two reactions have occurred, catalysed by: (1), a phosphoglucomutase and (2), an uridylyltransferase.

N-Acetamidoglucose-6-P → *N*-acetamidoglucose-1-P (1)

N-Acetamidoglucose-1-P + UTP → UDPacetamidoglucose + PP (2)

In both glands, the rate of UDPacetamidoglucose synthesis was of the same order as that for UDPgalactose but in the sublingual gland it was about twice as high as in the submaxillary one. With triphosphonucleosides other than UTP a very low activity was observed, not exceeding 1% of that found with UTP.

Formation of UDPaminoglucose was very small and the results were not reproducible.

Eight days after alloxan administration, the activity of all three uridylyltransferases in the salivary glands was decreased. However, the activities in the sublingual gland were much more affected. In the submaxillary gland, the decreases amounted to about 50% whereas in the sublingual gland they were greater, and formation of UDPgalactose was lower by as much as 93% (Table 5).

DISCUSSION

Dixit, Lowe & Lazarow (1962) have demonstrated that the content of insulin in β-cells of the islets of Langerhans decreased to 5% of the normal amount within 48 h after alloxan administration. In our experiments, the response to alloxan in rat salivary glands appeared on the 3rd day in the sublingual gland, and only on the 5th day in the submaxillary gland. The observed changes consisted in a decrease in the weight of the glands, associated with a decrease in the content of protein and bound sialic acids. The decrease of protein might be ascribed to insulin deficiency. Wool (1965) suggested that insulin, or its metabolism product, stimulates protein synthesis by initiating mRNA synthesis by binding with the repressor. He also considered the possibility that insulin could increase the permeability of intracellular membranes, thus facilitating the transport of RNA from cell nucleus. Further experiments of Wool & Cavicchi (1966, 1967) indicate that insulin affects protein biosynthesis at the translation level.

The suggestion put forward in the previous paper (Szymczyk & Jachimowicz, 1971) that salivary glands have a mechanism for the synthesis of the carbohydrate moiety of mucoprotein, was supported by demonstration of the presence of three enzymes involved in this pathway. In extracts from both glands, formation of UDPglucose from UTP and glucose-1-P, of UDPacetamidoglucose from UTP and *N*-acetamide-6-P, and of UDPgalactose from UDPglucose and galactose-1-P, was demonstrated. The activity of the enzymes catalysing the formation of UDPglucose was several-fold higher in the sublingual gland than in the submaxillary one. It should be noted that the sublingual gland is characterized by a much higher content of mucoprotein. In alloxan-diabetic rats, the activity of the enzymes studied was very much lower, especially in the sublingual gland.

The results of our experiments, which demonstrated that in salivary glands *in vitro* insulin stimulated mucoprotein synthesis (Szymczyk & Jachimowicz, 1971) and that in alloxan-diabetic rats there was a distinct decrease in the content of mucoprotein and in the activity of uridylyltransferases, point to the regulatory role of insulin in formation of mucoprotein in the sublingual and submaxillary glands.

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WPŁYW CUKRZYCY ALOKSANOWEJ NA ZAWARTOŚĆ KWASÓW SJALOWYCH I AKTYWNOŚĆ URYDYLILOTRANSFERAZ W GRUCZOŁACH ŚLINOWYCH SZCZURA

Streszczenie

1. W śliniance podżuchwowej i podjęzykowej szczura kwasy sjalowe występują tylko w formie związanej. W wyciągach z obu ślinianek wykazano aktywność urydylilotransferaz katalizujących powstawanie UDPglukozy, UDPgalaktozy i UDPacetyloglukozaminy.

2. U szczurów z cukrzycą aloksanową stwierdzono znaczny spadek wagi obu gruczołów z równoczesnym obniżeniem zawartości białka i kwasów sjalowych. Aktywność badanych enzymów była obniżona o ok. 50% w śliniance podżuchwowej i w dużo większym stopniu w śliniance podjęzykowej.

Received 26 November, 1970.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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α-NAPHTHYL RIBONUCLEOSIDE-3′ PHOSPHATE ANALOGUES FOR THE CYTOCHEMICAL AND COLORIMETRIC ASSAY OF RIBONUCLEASES

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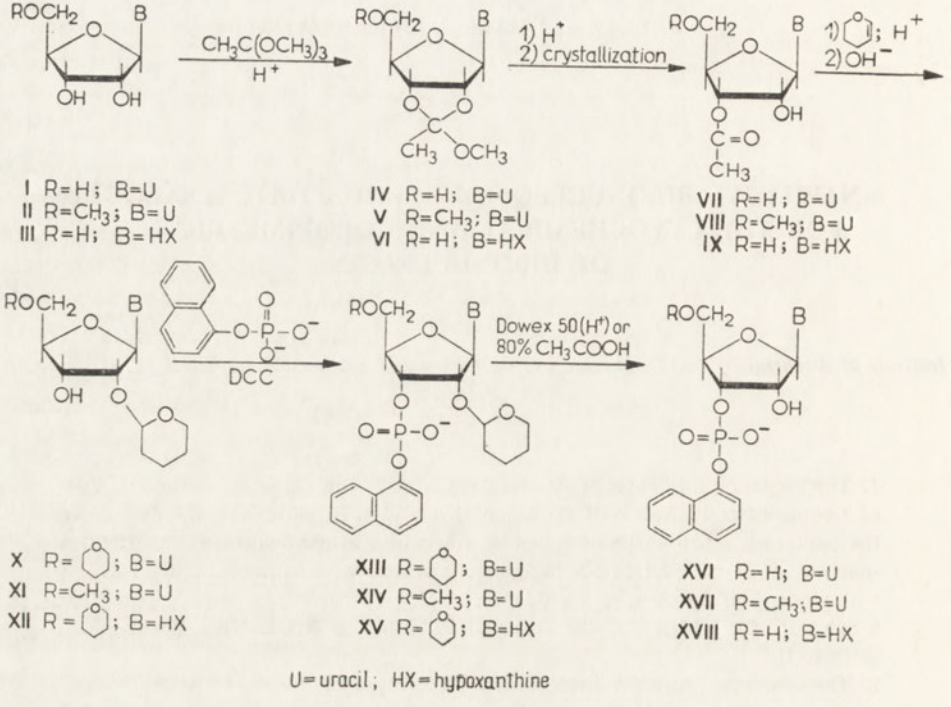
1. The preparation of three substrates for the histochemical and colorimetric assay of ribonucleases is described: (1) α-naphthyl uridine-3′ phosphate, a substrate for the pancreatic and T_2 RNases, slightly susceptible to phosphodiesterase II; (2) α-naphthyl 5′-*O*-methyluridine-3′ phosphate, a substrate for pancreatic and T_2 RNases with marked resistance to phosphodiesterase II; (3) α-naphthyl inosine-3′ phosphate, a substrate for RNase T_2 with some susceptibility to RNase T_1 and phosphodiesterase II.
2. The synthetic procedure involves the preparation of the respective 3′-*O*-acetylnucleosides, their tetrahydropyranylation to obtain nucleosides with protected 2′ and 5′ positions and subsequent condensation with naphthylphosphate in the presence of DCC. The products are isolated by preparative alumina thin-layer chromatography and DEAE-cellulose column chromatography.
3. The rates of hydrolysis of the substrates with pancreatic RNase, RNase T_1, RNase T_2 and spleen phosphodiesterase are presented and discussed in terms of substrate specificity.

The preparation of α-naphthyl uridine-3′ phosphate (XVI) and its application to the histochemical localization of alkaline ribonuclease was originally described by Sierakowska, Zan-Kowalczewska & Shugar (1965). The synthesis of other esters of ribonucleoside-3′ phosphates suitable for the assay of ribonuclease activity (Molemans, van Montagu & Fiers, 1968) and its histochemical localization (Wolf, Horwitz, Freisler, Vazquez & Von der Muehll, 1968) have since been reported.

The synthetic procedure for the preparation of α-naphthyl uridine-3′ phosphate (XVI) has now been modified to yield a product of higher purity. Furthermore, an analogous compound, α-naphthyl inosine-3′ phosphate (XVIII), hydrolysed rapidly

by RNase T_2 * and very slowly by RNase T_1, but resistant to pancreatic RNase, has been prepared. The latter compound has been found useful in the histochemical localization and colorimetric assay of plant nucleases (Sierakowska & Shugar, in preparation). However, both of these were hydrolysed, albeit at an extremely slow rate, by splenic PDase II. Since this might, in some cases, limit the specificity of these substrates for RNases, the introduction of a 5′-*O*-protective group was conceived



as a means of providing resistance to PDase II (Sierakowska & Shugar, 1971). Hence, α-naphthyl-5′-*O*-methyluridine-3′ phosphate (XVII), an RNase substrate, relatively resistant to splenic PDase II, has been prepared as the first in a series of such analogues.

The present paper describes procedures for the preparation of naphthyl substrates for RNases as well as some data on their specificities.

Preparation of substrates

Preparation of α-naphthyl ribonucleoside-3′ phosphate involved the selective phosphorylation of the 3′-*O*-position, and hence required the protection of the

* The following abbreviations are used: PDase I, phosphodiesterase I (EC 3.1.4.1); PDase II, phosphodiesterase II (EC 3.1.4.1); RNase, ribonuclease (EC 2.7.7.16); RNase T_1, guanyloribonuclease from *Aspergillus oryzae* (EC 2.7.7.26); RNase T_2, non-specific ribonuclease from *Aspergillus oryzae* (EC 2.7.7.17); Tp-nitrophenyl, *p*-nitrophenyl thymidine-3′ phosphate; DCC, *N,N′*-dicyclohexylcarbodiimide; TLC, thin-layer chromatography.

2′,5′-*O*-positions of the nucleoside. Protection of the 2′-hydroxyl function also proved useful in the isolation and storage of the α-naphthyl ribonucleoside-3′ phosphates. The latter compounds are somewhat unstable and, in alkaline media, undergo spontaneous decomposition with concomitant formation of nucleoside-2′,3′-cyclic phosphates and free naphthol. The presence of a 2′-*O*-protecting group prevents such decomposition. The lability of α-naphthyl ribonucleoside-3′ phosphates in alkali called for an alkali-stable 2′-*O*-protecting group, removable under relatively mild conditions. The tetrahydropyranyl group fulfils this requirement and, moreover, proved sufficiently stable to survive the procedure for isolation of the product.

2′,5′-Di-*O*-tetrahydropyranylnucleosides (X, XII) were obtained from 3′-*O*--acetylnucleosides prepared according to the procedure of Fromageot, Griffin, Reese & Sulston (1967). This involved the preparation of 2′,3′-*O*-methoxyethylidene-nucleosides (IV, VI) which, upon mild acid hydrolysis, followed by crystallization, yielded the 3′-*O*-acetylnucleosides (VII, IX). These were treated with dihydropyran according to Griffin, Jarman & Reese (1968) to obtain the 2′,5′-di-*O*-tetrahydropyranyl-3′-*O*-acetylnucleosides. The 3′-*O*-acetyl groups were removed by treatment with ammonia to give the required 2′,5′-di-*O*-tetrahydropyranylnucleosides (X, XII).

The protected nucleoside for the preparation of α-naphthyl 5′-*O*-methyluridine--3′ phosphate (XVII) was prepared by essentially the same procedure, yielding the crystalline 5′-*O*-methyl-3′-*O*-acetyluridine (VIII) and, finally, 5′-*O*-methyl-2′-*O*--tetrahydropyranyluridine (XI).

The protected nucleosides were subsequently phosphorylated with naphthylphosphate in the presence of DCC. Following phosphorylation, the products were isolated by preparative alumina TLC which effectively removed the contaminating naphthylphosphate. Since removal of naphthylphosphate was of prime importance, its absence at this stage was ascertained by incubating a sample of the product with *E. coli* phosphatase. The products were further purified by DEAE-cellulose column chromatography.

The purified tetrahydropyranylated substrates were fully stable when stored over P_2O_5. α-Naphthyl nucleoside-3′ phosphates (XVI - XVIII) were obtained from these as desired by two alternative procedures, (a) treatment with acetic acid or, (b) with Dowex 50 (H^+ form). The latter was found preferable because of its simplicity. The unprotected substrates were stable for many weeks when deep-frozen in aqueous solutions adjusted to pH 5 - 6. If trace decomposition occurred, the released naphthol was readily extracted with ether.

DEAE-cellulose chromatography was essential for removal of traces of unknown products which released naphthylphosphate upon treatment with acid. The heterocyclic base origin of this naphthylphosphate contaminant was established by phosphorylation of 2′,3′,5′-tri-*O*-methyluridine with naphthylphosphate in the presence of DCC. A small percentage of the 2′,3′,5′-tri-*O*-methyluridine was in fact found to bind the naphthylphosphate in an acid-labile linkage (cf. Jacob & Khorana, 1965).

Other methods of synthesis were examined in order to avoid substitution of naphthylphosphate in an acid-labile linkage. However, condensation of 2′,5′-di-*O*--tetrahydropyranyluridine-3′ phosphate with α-naphthol in the presence of DCC

failed to take place. A similar condensation in pyridine with the aid of mesitylenesulfonyl chloride (Jacob & Khorana, 1964) gave the desired product, but in very low yield. Phosphorylation of the protected nucleoside with di-α-naphthylphosphoryl chloride (less likely to participate in side reactions), in pyridine or in acetonitrile in the presence of 2,4-lutidine (Ukita & Hayatsu, 1962; Reese & Saffhill, 1968), was unsuccessful. Treatment of the nucleoside with di-α-naphthylphosphate in the presence of DCC similarly failed to result in condensation. An identical reaction with mesitylenesulfonyl chloride as the condensing agent gave the desired product, but in very low yield; moreover, the compound bearing the acid-labile naphthylphosphate still contaminated the reaction product. The contamination persisted also in the product obtained by phosphorylating the protected nucleoside with α-naphthylphosphoryl dichloride.

Specificity of substrates

Table 1 gives the respective rates of hydrolysis of the substrates by pancreatic RNase, RNase T_1, RNase T_2 and PDase II. The degrees of purity of the enzyme preparations varied appreciably, making any direct comparison between their rates of hydrolysis of a given substrate meaningless. Therefore, the activities of these enzyme preparations against their optimal substrates, RNA for the ribonucleases and *p*-nitrophenyl thymidine-3′ phosphate for PDase II, are included for comparative purposes.

α-Naphthyl uridine-3′ phosphate is hydrolysed by pancreatic RNase and RNase T_2. Its rate of hydrolysis by pancreatic RNase is 1% that for the hydrolysis of RNA by the same enzyme preparation. RNase T_2 hydrolyses this compound at about 3-fold the rate for RNA hydrolysis. α-Naphthyl uridine-3′ phosphate is fully resistant to RNase T_1, but is hydrolysed by splenic PDase II at 0.3% the rate for *p*-nitrophenyl thymidine-3′ phosphate.

Table 1

Activities of pancreatic RNase, RNase T_1, RNase T_2 and PDase II towards the various α-naphthyl ribonucleoside-3′ phosphates in relation to their activities towards their optimal substrates

Conditions of assay are given in General Methods.

Substrate	Pancreatic RNase	RNase T_1	RNase T_2	PDase II
	μmoles substrate hydrolysed/h/mg protein			
RNA	500 000	115 000	154	
p-Nitrophenyl thymidine-3′ phosphate				350
α-Naphthyl uridine-3′ phosphate	5 000	<0.05	419	1
α-Naphthyl 5′-*O*-methyluridine-3′ phosphate	3 000	<0.05	783	0.07
α-Naphthyl inosine-3′ phosphate	<0.4	25	1420	0.7

Substitution of the 5′-OH by 5′-OCH_3 in α-naphthyl uridine-3′ phosphate hardly affects its susceptibility to RNase, but leads to a 15-fold reduction in rate of hydrolysis by PDase II (Sierakowska & Shugar, 1971); so that α-naphthyl 5′-*O*--methyluridine-3′ phosphate is more specific towards pancreatic and T_2 RNases than α-naphthyl uridine-3′ phosphate.

α-Naphthyl inosine-3′ phosphate appears fully resistant to pancreatic ribonuclease. It is hydrolysed by RNase T_2 at 9-fold the rate for RNA. The rate of its hydrolysis by RNase T_1 is, as expected, extremely slow, i.e. only 0.02% that for the hydrolysis of RNA by the same enzyme preparation. It is attacked by splenic PDase at 0.2% the rate for *p*-nitrophenyl thymidine-3′ phosphate.

Table 1 shows that none of the substrates exhibit absolute specificity: α-naphthyl uridine-3′ phosphate is a reasonably good substrate for pancreatic RNase and a very good one for RNase T_2; it may also be hydrolysed by PDase II but this disadvantage can largely be overcome by the use of its 5′-*O*-methyl analogue; α-naphthyl inosine-3′ phosphate is a very good substrate for RNase T_2 and a poor one for RNase T_1 and PDase II.

Due to these inherent limitations in substrate specificity, the type of ribonuclease activity assayed can be established only by correlation of results obtained with two substrates. Activity against α-naphthyl 5′-*O*-methyluridine-3′ phosphate and lack of activity against the inosine substrate is an indication of pancreatic ribonuclease. Activity against both substrates indicates RNase T_2. Activity against α-naphthyl inosine-3′ phosphate and lack of activity against α-naphthyl uridine-3′ phosphate point to RNase T_1 activity.

EXPERIMENTAL

Materials and general methods

Paper chromatography was performed using the ascending technique on Whatman no. 1 paper except where otherwise noted. The solvent systems used were: *A*, *n*--butanol - water (86:14, v/v); *B*, ethanol - 1 M-ammonium acetate, pH 7.5 (5:2, v/v); *C*, isopropanol - concentrated ammonia - water (7:1:2, by vol.).

The course of synthetic reactions was followed by TLC on microscopic slides coated with Merck TLC Silica Gel GF_{254}. The chromatograms were developed in chloroform containing 5, 10 and 15% methanol (v/v) — solvent *D*, *E*, *F*, respectively.

Preparative TLC chromatography was performed on plates coated with Merck Aluminum Oxide PF_{254} (30 g of gel per 16 cm × 20 cm plate per 5000 OD_{260} units of material). The chloroform used in column chromatography was extracted with water, dried over calcium chloride and redistilled. Column chromatography on silicic acid was performed using Mallinckrodt CC7 silicic acid. DEAE-cellulose (carbonate form) and triethylammonium bicarbonate buffer were prepared according to Khorana & Connors (1966).

All evaporations were carried out using a rotary evaporator under reduced pressure at a temperature not exceeding 37°C.

All the solvents used in syntheses were dried according to standard methods and redistilled under anhydrous conditions. α-Naphthylphosphoryl dichloride and α-naphthylphosphoric acid were prepared according to Friedman & Seligman (1950).

Crystalline pancreatic RNase was a Reanal (Budapest, Hungary) product stabilized in solution with 0.1% gelatin. Crystalline RNase T_1 from *Aspergillus oryzae*, grade I, was a Sigma Chem. Co. (St. Louis, Mo., U.S.A.) product. RNase T_2 was a gift from Professor F. Egami; PDase II ex spleen was a purified preparation generously donated by Dr. G. Bernardi and was stabilized in solution according to Bernardi & Bernardi (1968); venom phosphodiesterase I (VPH) and *E. coli* alkaline phosphatase (BAPF) were Worthington Biochem. Co. (Freehold, N.J., U.S.A.) preparations.

Enzyme assays. The relative rates of activity of RNase and PDase II against the naphthyl substrates were determined by incubations for 30 min at 37°C at 10 mM substrate concentrations in a total volume of 0.05 ml. The following incubation media were used: 0.1 M-phosphate buffer, pH 7.4, for pancreatic RNase; 0.07 M--acetate buffer, pH 4.5, 3 mM with respect to EDTA for RNase T_2; 0.07 M-tris-HCl buffer, pH 7.5, 3 mM with respect to EDTA for RNase T_1; 0.1 M-ammonium acetate, pH 5.7, 0.1% with respect to Tween 80 and 0.01 M with respect to EDTA, for PDase II. The naphthol liberated was assayed as described previously (Sierakowska & Shugar, 1971). The activities of RNases against RNA were estimated according to Beard & Razzell (1964) using highly polymerized yeast RNA in the media indicated above. PDase II activity against Tp-nitrophenyl was assayed according to Razzell & Khorana (1961).

Preparation of α-naphthyl 2',5'-di-O-tetrahydropyranyluridine-3' phosphate (XIII)

3'-O-Acetyluridine (VII) was prepared according to Fromageot *et al.* (1967).

2',5'-Di-O-tetrahydropyranyluridine (X). 3'-*O*-Acetyluridine (3.5 g), toluene-*p*--sulfonic acid monohydrate (605 mg) and dry dihydropyran (23.4 ml) were stirred magnetically in dry dioxane (120 ml) with the exclusion of moisture. After one hour, TLC with solvent *D* revealed the absence of 3'-*O*-acetyluridine, R_F 0.1, and the appearance of 3'-*O*-acetyl-2',5'-di-*O*-tetrahydropyranyluridine, R_F 0.48. The reaction mixture was cooled in an ice bath and 100 ml of concentrated ammonia-methanol (1:1, v/v) added. The total was taken to dryness and treated overnight with concentrated ammonia - methanol (1:1, v/v). The mixture was then evaporated to a yellow glass, taken up in chloroform and filtered. The filtrate was taken to dryness and the residue dissolved in water. The aqueous solution was extracted with ether (3×20 ml) and the ether layer washed with water. All the aqueous solutions were combined, evaporated and dried over P_2O_5. The residue was dissolved in chloroform and chromatographed on a column (9 cm^2 × 20 cm) of silicic acid. The column was washed with chloroform, which removed some impurities (R_F 0.95 on TLC with solvent *D*). The required product (R_F 0.80 in solvent *A*, R_F 0.30 and 0.33 for the mixture of diastereoisomers on TLC with solvent *D*) was eluted with 1.5 l chloroform containing 2% methanol, concentrated to a colourless foam and dried over P_2O_5. The yield was 4.25 g (85% theoretical).

α-Naphthyl 2′,5′-di-O-tetrahydropyranyluridine-3′ phosphate (XIII). α-Naphthylphosphoric acid (390 mg) was repeatedly evaporated with dry pyridine, combined with 2′,5′-di-*O*-tetrahydropyranyluridine (80 mg) rendered anhydrous in the same manner, and dissolved in dry pyridine (5 ml); DCC (675 mg) was added and the mixture kept in the dark for 18 h. TLC with solvent *E* indicated the disappearance of the diastereoisomers of 2′,5′-di-*O*-tetrahydropyranyluridine. The reaction mixture was then cooled in a dry ice-methanol bath and 3 ml of 50% aqueous pyridine were added. After 1 h at room temperature, the total was evaporated to dryness and taken up in water adjusted to pH 9 with ammonia. The aqueous solution was filtered, extracted with ether (4×10 ml), concentrated to a small volume (2 ml) and subjected to preparative chromatography on alumina, developed in 50% aqueous methanol adjusted to pH 9. The required compound (R_F 0.50) was eluted with 50% aqueous methanol taken to dryness and dissolved in 50 ml of water adjusted to pH 9. The total was chromatographed on a DEAE-cellulose (carbonate form) column (2 cm^2 × ×30 cm). The column was washed with water and then eluted with 0.01 M-triethylammonium bicarbonate at a flow rate of 1.5 ml/min, fractions being collected at 15 min intervals. The effluent was taken to dryness and the residue lyophilized to remove traces of triethylammonium bicarbonate. The yield of product (R_F 0.56 in solvent *A*, R_F 0.97 in solvent *B*) was 112 mg (93% theoretical). λ_{max} (water) 265 mμ, ε_{max} 1.21×10^4.

Preparation of α-naphthyl 5′-O-methyl-2′-O-tetrahydropyranyluridine-3′ phosphate (XIV)

5′-O-Methyluridine (II) was prepared by Mr. J. Kuśmierek according to a procedure described by Kuśmierek and Shugar (in preparation).

5′-O-Methyl-3′-O-acetyluridine (VIII). To 5′-*O*-methyluridine (150 mg) were added dry trimethylorthoacetate (3 ml) and toluene-*p*-sulfonic acid monohydrate (30 mg) and the suspension stirred for 2 h under anhydrous conditions. TLC with solvent *E* showed only a trace of 5′-*O*-methyluridine, R_F 0.24, and the desired 5′-*O*-methyl-2′,3′-methoxyetylideneuridine, R_F 0.66. The total was taken to dryness and treated with 3 ml of 5% aqueous acetic acid for a few minutes. The acetic acid was then removed by repeated evaporation with absolute ethyl alcohol and the residue dried overnight over P_2O_5. The product was dissolved in chloroform and chromatographed on a column (3 cm^2 × 12 cm) of silicic acid. The column was washed with chloroform and 5′-*O*-methyl-2′(3′)-*O*-acetyluridine was eluted with chloroform containing 2% methanol. The product was taken to dryness and 5′-*O*-methyl-3′-*O*-acetyluridine crystallized from ethyl acetate. The yield was 105 mg (70% theoretical), m.p. 150 - 153°C. The product had R_F 0.49 on TLC with solvent *E* and R_F 0.66 in solvent *A*.

5′-O-Methyl-2′-O-tetrahydropyranyluridine (XI). 5′-*O*-Methyl-3′-*O*-acetyluridine (60 mg) and toluene-*p*-sulfonic acid monohydrate (13 mg) dissolved in 2 ml dry dioxane was treated with dry dihydropyran (0.4 ml) and the total stirred magnetically for 40 min. TLC with solvent *E* showed only the tetrahydropyranyl derivative

R_F 0.83. 5 ml of methanol - concentrated ammonia (1:1, v/v) was then added to the reaction mixture cooled in an ice bath. The total was then evaporated to a light yellow syrup. The syrup was extracted with chloroform (4×2 ml), and the combined extracts filtered and taken to dryness. The residue was treated overnight at room temp. with 5 ml methanol - conc. ammonia (1:1, v/v), taken to dryness and dried over P_2O_5. The resultant glass was dissolved in chloroform and chromatographed on a column (3 cm^2×15 cm) of silicic acid. The column was washed with chloroform to remove impurities. The desired product was eluted with chloroform containing 2% methanol, taken to dryness and stored over P_2O_5. The yield of the two diastereoisomers of 5′-*O*-methyl-2′-*O*-tetrahydropyranyluridine (R_F 0.55 and 0.62 on TLC with solvent *E*) was 49 mg (71% theoretical).

*α-Naphthyl 5′-*O*-methyl-2′-*O*-tetrahydropyranyluridine-3′ phosphate (XIV).* To a solution of *α*-naphthylphosphoric acid (170 mg) in 2 ml dry pyridine was added 5′-*O*-methyl-2′-*O*-tetrahydropyranyluridine (49 mg). The mixture was rendered anhydrous by repeated evaporation from dry pyridine and dissolved in 3 ml dry pyridine. DCC (300 mg) was added, the reaction mixture kept in the dark for 24 h, 2 ml 50% aqueous pyridine added to the reaction mixture cooled in dry ice - methanol and after 30 min the total was taken to dryness. The residue was taken up in 50 ml of water, the aqueous extract rapidly adjusted to pH 9.0, filtered, and extracted with ether. During all subsequent operations the solutions were maintained at pH 9. The filtrate was concentrated by evaporation to about 0.5 ml and preparative chromatography on alumina performed with 50% aqueous methanol. The product, R_F 0.50, was eluted with 50% aqueous methanol and taken to dryness. It was then dissolved in 50 ml of water, adjusted to pH 9 with ammonia, and chromatographed on a DEAE-cellulose (carbonate form) column (2 cm^2×30 cm). The column was washed with water and subsequently eluted with 0.008 M-triethylammonium bicarbonate at a flow rate of 1.5 ml/min, fractions being collected at 15 min intervals. The effluent was taken to dryness and the residue lyophilized to remove traces of triethylammonium bicarbonate. The yield (R_F 0.50 in solvent *A*, R_F 0.97 in solvent *B*) was 75 mg (75% theoretical); λ_{max} (water) 265 mμ, ε_{max} 1.21×10^4.

*Preparation of α-naphthyl 2′,5′-di-*O*-tetrahydropyranylinosine-3′ phosphate (XV)*

*3′-*O*-Acetylinosine (IX)* was obtained by a modification of the procedure of Fromageot *et al.* (1967). Inosine (4 g) dissolved in dry dimethylformamide (20 ml) was treated with trimethylorthoacetate (25 ml) and trifluoroacetic acid (0.6 ml) for 2 h with magnetic stirring. Chromatography on Eastman-Kodak F cellulose plates in solvent *A* showed only a trace of free inosine. The mixture was taken to dryness, treated with 10% acetic acid (50 ml) for 30 min, again taken to dryness and dried over P_2O_5. The resultant glass was suspended in 100 ml of chloroform, mixed with silicic acid and the total applied to a column (9 cm^2×25 cm) of silicic acid. The column was washed with chloroform, followed by chloroform containing 4% methanol, which removed all side products having R_F values higher than that of 2′(3′)-*O*-acetylinosine (TLC, R_F 0.30). The desired product was eluted with chloroform

containing 6% methanol. The yield was 2.7 g (58% theoretical). 3′-*O*-Acetylinosine was crystallized from absolute ethyl alcohol, yielding 1.76 g (38% theoretical), m.p. 200 - 202°C. The product had R_F 0.32 in solvent *A* and R_F 0.30 on TLC with solvent *F*.

2′,5′-Di-O-tetrahydropyranylinosine (XII). 3′-*O*-Acetylinosine (1.4 g), toluene-*p*--sulfonic acid monohydrate (460 mg), dihydropyran (9 ml) and dry dimethylformamide (5 ml) were stirred magnetically in dry dioxane (60 ml) under anhydrous conditions for about 2 h to give a clear solution. TLC indicated the disappearance of starting material and the presence of the desired product (R_F 0.70 in solvent *F*) in addition to some impurities (R_F 0.95). The mixture was cooled, 50 ml of methanol - - concentrated ammonia (1:1, v/v) added and the total taken to dryness. The residue was taken up in chloroform, filtered and taken to dryness. The residue was treated overnight with 50 ml methanol - concentrated ammonia (1:1, v/v), taken to dryness, dissolved in 50 ml water and extracted with ether (3×15 ml). The ether layer was washed with water (2×10 ml) and the combined aqueous solutions were taken to dryness and dried over P_2O_5. The residue was dissolved in chloroform and chromatographed on a column (9 cm²×20 cm) of silicic acid. The impurities were removed with chloroform and the desired product was eluted with chloroform containing 2% methanol. The product was taken to dryness and stored over P_2O_5. The yield was 1.22 g (62% theoretical). A mixture of crystalline diastereoisomers could be obtained by crystallization from chloroform - ether, m.p. 164 - 172°C, R_F 0.78 in solvent *A*, R_F 0.61 and 0.65 for the mixture of diastereoisomers on TLC with solvent *F*.

α-Naphthyl 2′,5′-di-O-tetrahydropyranylinosine-3′ phosphate (XV). To a solution of α-naphthylphosphoric acid (3.2 g) in 15 ml dry pyridine was added 2′,5′-di-*O*--tetrahydropyranylinosine (1.17 g). The mixture was rendered anhydrous by repeated evaporations of dry pyridine and dissolved in 25 ml dry pyridine. DCC (5.5 g) was then added and the reaction mixture kept sealed in the dark for 48 h. TLC with solvent *F* indicated the disappearance of the diastereoisomers of 2′,5′-di-*O*-tetrahydropyranylinosine. 5 ml 50% aqueous pyridine was then added to the reaction mixture cooled in dry ice - methanol and, after 1 h, the total taken to dryness. The residue was taken up in water, rapidly adjusted to pH 9.0 and filtered. During all subsequent operations the solutions were maintained at pH 9. The filtrate was extracted with ether, concentrated to a small volume and preparative chromatography on alumina was performed in 50% aqueous methanol. The band, R_F 0.50, containing the desired product was eluted with 50% aqueous methanol. The eluate was taken to dryness, dissolved in 1 l of water, adjusted to pH 9 with ammonia and chromatographed on a DEAE-cellulose (carbonate form) column (12.3 cm²× ×60 cm). The column was washed with water and then eluted with 0.02 M-triethylammonium bicarbonate at a flow rate of 1.5 ml/min, fractions being collected at 15 min intervals. The effluent was taken to dryness and the residue lyophilized to remove traces of triethylammonium bicarbonate. The yield of product (R_F 0.53 in solvent *A*, R_F 0.97 in solvent *B*) was 1 g (58% theoretical); λ_{max} (water) 249 mμ, ε_{max} 1.16×10^4.

Removal of protective groups

(*a*) *Acetic acid treatment.* 50 mg of protected substrate was treated with 1 ml of 80% acetic acid for 3 h at room temperature. The acetic acid was removed by repeated evaporations with water, the residue taken up in 2 ml of water and extracted with ether (2×1 ml). The aqueous solution was adjusted to pH 5 - 6 and stored in the freezer. If the sodium salt of the product was desired, the solution was passed through Dowex 50 (Na^+ form) just prior to extraction with ether.

(*b*) *Dowex 50 (H^+ form) treatment.* To 50 mg of the protected substrate dissolved in 2 ml of water was added 100 mg of Dowex 50 (H^+ form). After 30 min the supernatant was decanted and carefully adjusted to pH 5 - 6 with 1 M-sodium hydroxide. It was subsequently extracted with ether (2×1 ml) and stored in the freezer.

Removal of tetrahydropyranyl protective groups may be checked by chromatography on Eastman Kodak F Cellulose plates developed in solvent *A*. Chromatography indicates the complete disappearance of the protected compounds, R_F 0.83, 0.83 and 0.72 for the uridine, 5′-*O*-methyluridine and inosine derivatives, respectively, and the appearance of a spot corresponding to α-naphthyl nucleoside-3′ phosphate (R_F 0.44, 0.55 and 0.33, respectively). The substrate concentration is estimated by spectrophotometry.

Enzymic substrate purity tests

The lack of contamination of the substrates with α-naphthylphosphate or α-naphthyl nucleoside-5′ phosphate was ascertained by incubation of a sample with *E. coli* alkaline phosphatase or venom phosphodiesterase. To 1 μmole of naphthol-free substrate was added 0.1 ml of water, 0.1 ml of 0.2 M-tris-HCl buffer, pH 8.0, and 0.05 ml of a freshly prepared 1% aqueous solution of Fast Red TR. The faintly pink mixture was rapidly divided into three parts and placed in wells in a porcelain plate. One μl of *E. coli* phosphatase was added to one well, 10 μg of venom phosphodiesterase to the second, the third serving as control. Identical colour development in the test solution and control after 5 min incubation at room temperature indicated lack of contamination with the respective substrate.

The homogeneity of the substrates was controlled by incubation with an excess of the appropriate ribonuclease followed by chromatography of the products. The substrates were quantitatively converted into the respective nucleoside-3′ phosphates and free naphthol, confirming that the crystalline mono-*O*-acetylnucleosides used in the synthesis were exclusively the 3′-*O*-acetyl isomers.

We are deeply indebted to Dr. D. Shugar for his guidance; to Dr. C. B. Reese for suggestion and helpful discussions as well as samples of acetylated nucleosides; to Dr. G. Bernardi for his purified phosphodiesterase II; to Dr. F. Egami for RNase T_2; to Mr. Jarosław Kuśmierek for the 2′,3′,5′-tri-*O*-methyluridine and for the

5′-*O*-methyluridine used for the preparation of the α-naphthyl ester; and to Mrs. Halina Szemplińska for excellent technical assistance. This investigation was supported by the Wellcome Trust, the World Health Organization and the Agricultural Research Service, U.S. Dept. of Agriculture.

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ANALOGI 3′-α-NAFTYLOFOSFORANÓW RYBONUKLEOZYDÓW DO CYTOCHEMICZNYCH I KOLORYMETRYCZNYCH OZNACZEŃ RYBONUKLEAZ

Streszczenie

1. Opisano metodę syntezy trzech substratów do histochemicznych i kolorymetrycznych oznaczeń RNaz:
1) 3′-α-naftylofosforan urydyny, substrat dla RNazy trzustkowej i RNazy T_2 charakteryzujący się niewielką podatnością na działanie fosfodwuesterazy II;
2) 5′-*O*-metylo-3′-α-naftylofosforan urydyny, substrat dla powyższych RNaz charakteryzujący się zwiększoną opornością na działanie fosfodwuesterazy II;
3) 3′-α-naftylofosforan inozyny, substrat dla RNazy T_2, posiadający niewielką podatność na działanie RNazy T_1 i fosfodwuesterazy II.

2. Substraty otrzymano przez kolejne przejścia: acetylację grupy 3′-OH odpowiedniego nukleozydu, blokowanie pozostałych grup przy użyciu dwuhydropyranu i kondensację zdezacetylowanego nukleozydu z α-naftylofosforanem w obecności *N*,*N*′-dwucykloheksylokarbodwuimidu. Produkty wydzielono za pomocą chromatografii cienkowarstwowej na Al_2O_3 i chromatografii kolumnowej na DEAE-celulozie.

3. Ustalono szybkości hydrolizy substratów przez RNazę trzustkową, T_1, T_2 oraz fosfodwuesterazę II i omówiono je z punktu widzenia specyficzności substratów.

Received 6 January, 1971.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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NICOTINAMIDE DINUCLEOTIDES IN MICROORGANISMS PRODUCING PEPTIDE AND MACROLIDE ANTIBIOTICS

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1. Participation of NADPH in biosynthesis of macrolide antibiotics: erythromycin and polyene tetraene antibiotics is manifested by a distinct decrease in the NADPH/ /$NADP^+$ ratio at the outset of biosynthesis of these antibiotics during growth of *Streptomyces erythreus* and *S. noursei* var. *polifungini*. 2. In *S. griseus* the reduced forms of both nucleotides accumulate during biosynthesis of the peptide antibiotic, viomycin, whereas in *Bacillus subtilis* producing bacitracin, another peptide antibiotic, the oxido-reduction state of nicotinamide dinucleotides remains unchanged. 3. A very high activity of NAD glycohydrolase (EC 3.2.2.6) in *S. griseus*, exceeding that of *Neurospora crassa*, is associated with the predominance (98%) of the reduced forms in the nicotinamide nucleotide pool. In the other investigated strains the activity of this enzyme is negligible.

The nicotinamide dinucleotide profile in microorganisms producing antibiotics reflects the energy state due to secondary metabolism of differentiating cells in the stationary phase of growth. It illustrates also the role of these nucleotides in biosynthesis of antibiotics.

Composition of the pool of nicotinamide coenzymes has been investigated in *Streptomyces griseus* and *Bacillus subtilis* producing peptide antibiotics: viomycin and bacitracin, and in *Streptomyces erythreus* and *Streptomyces noursei* producing macrolide antibiotics: erythromycin and tetraene antibiotics of nystatin type. In biosynthesis of the latter compounds NADPH serves as a reducing agent in the mechanism similar to that involved in biosynthesis of fatty acids (Corcoran & Chick, 1966).

MATERIALS AND METHODS

Reagents. NAD^+, $NADP^+$ and tris were obtained from C. F. Boehringer Soehne (Mannheim, West Germany); bovine blood serum albumin was a product of Serva (Heidelberg, West Germany) and glucose-6-phosphate and phenazine methosulphate

of Sigma (St. Louis, Mo., U.S.A.); Difco yeast extract and Bacto meat extract were from Difco Laboratories (Detroit, Mich., U.S.A.) and peptone from Bacutil (Warszawa, Poland). Other reagents used were from Fabryka Odczynników Chemicznych (Gliwice, Poland). Glucose-6-phosphate dehydrogenase (3.4 units/ml) was prepared from dried brewer's yeast after Kornberg (1955) and alcohol dehydrogenase ($13 \times \times 10^4$ units/ml) from baker's yeast according to Racker (1955).

Organisms. Highly productive strains synthesizing antibiotics were obtained from the Department of Microbiology, Institute of Antibiotics, Warsaw: *Streptomyces sp.* producing viomycin classified according to Waksman (1961) as *S. griseus* and its non-productive mutant, from dr. M. Tyc; *S. erythreus* producing erythromycin, from dr. B. Ostrowska-Krysiak; *B. subtilis* synthesizing bacitracin, from Mrs. D. Styczyńska (M.Sc.) and *S. noursei* var. *polifungini* ATTC 21 581 producing tetraene polyene antibiotics from Mrs. D. Kotiuszko (M.Sc.).

Media and culture conditions. All the strains were grown in 500 ml Erlenmayer flasks on a reciprocating shaker (240 rev./min) at 28°C (*S. noursei* and *S. griseus*) or 33°C (*S. erythreus* and *B. subtilis*) on the complex soluble media. The composition of the medium for *S. erythreus* was as follows: 100 ml of water; soya flour extract, 3 g; NaCl, 5 g; $(NH_4)_2SO_4$, 3 g; $CaCO_3$, 6 g; and in some experiments propanol, 0.8 ml. The medium for *S. griseus* was of the following composition: water, 100 ml; glucose, 5 g; Difco yeast extract, 0.5 g; soya flour extract, 1.5 g; $(NH_4)_2SO_4$ 0.6 g; $CaCO_3$, 0.5 g; soya oil, 2 g. *B. subtilis* was grown according to Shimura, Sasaki & Sugawara (1964), on the medium composed of soluble starch, peptone, meat extract and NaCl (1 g, 1 g, 0.5 g, 0.05 g, respectively per 100 ml of water). The alternative medium for *B. subtilis* had the following composition: water, 100 ml; soluble starch, 2.0 g; soya flour extract, 0.5 g; $CaCO_3$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.33 g; $MnCl_2$, 0.02 g. *S. noursei* was cultivated according to Kotiuszko, Morawska & Siejko (unpublished) on the media containing either oil (3 g/100 ml) or soluble starch and glucose (4 and 2.5 g, resp./100 ml); the other components of both media were as follows: water, 100 ml, corn steep liquor, 1 g; $CaCO_3$, 0.8 g; $(NH_4)_2SO_4$, 0.4 g; and sonicated brewer's yeast, 0.3 g. The yield of antibiotic on both media was practically the same.

Extraction of nicotinamide dinucleotides. The mycelium was collected from 3 - 5 flasks, spun down at 10 000 r.p.m. for 15 min and washed twice with cold distilled water; for determination of NAD^+ and $NADP^+$ the mycelium was extracted with 0.7 N-HCl in a glass homogenizer (17:3 w/v), and for the assay of NADH and NADPH with 1 N-KOH in 50% ethanol for 3 min in boiling water followed by homogenization at room temperature. The extracts were centrifuged at 10 000 r.p.m., neutralized to pH 7.0 and recentrifuged. The recovery of nucleotides added to mycelium was 97% in the case of oxidized and 90% with the reduced ones.

Determination of nicotinamide dinucleotides. The reduced and oxidized forms of nicotinamide nucleotides were estimated with oxygen electrode according to Greenbaum, Clark & McLean (1965); alcohol dehydrogenase and glucose-6-phosphate dehydrogenase were used as the specific reducing systems for NAD^+ and $NADP^+$, and phenazine methosulphate as the electron acceptor. The standard curves for

NAD^+ and $NADP^+$ were constructed over the range 0.1 - 0.6 and 0.1 - 1.0 µg, respectively.

Determination of NAD glycohydrolase activity was based on measurements of NAD decomposition by the cyanide method of Ciotti & Kaplan (1955).

Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951).

RESULTS AND DISCUSSION

The total pool of nicotinamide dinucleotides was lowered 2-fold in the stationary phase of growth of *B. subtilis* producing bacitracin but it was increasing significantly during growth of streptomyces (Table 1) both in species producing macrolide antibiotics (*S. erythreus*, *S. noursei*) and that synthesizing a peptidic viomycin (*S. griseus*).

To see whether these differences could be attributed to the enzymic systems catabolizing nicotinamide nucleotides, the activity of NAD glycohydrolase (EC 3.2.2.6) was determined in the investigated strains. A slight activity of this enzyme was observed both in *S. erythreus* and *B. subtilis* (appr. 0.001 µmol/mg protein/5 min) despite the opposite changes in the size of the nucleotide pool during growth of these bacteria. A very high activity of NAD glycohydrolase was, however, found in *S. griseus* producing viomycin (Table 2); this activity was higher than that of the Zn-requiring mutant of *Neurospora crassa* (Nason, Kaplan & Colowick, 1951), considered so far as the richest source of NAD glycohydrolase. The activity of this

Table 1

The pool of nicotinamide dinucleotides in microorganisms producing macrolide and polypeptide antibiotics

The values, µg/g wet wt., are averages of 3 - 7 experiments. *F*, variance ratio; L.S.D., least significant difference.

Strain and medium	Growth (h)				*F*	L.S.D. *P* 0.05
	24	48	72	96		
S. erythreus						
non-supplemented with PrOH	55.8	87.1	71.6	74.5	3.40[b]	20.6
supplemented with PrOH	47.7	51.8	57.6	57.3	1.34	11.1
S. noursei var. *polifungini*						
Carbon source: carbohydrates	34.8	52.7	55.6	57.5	7.44[c]	12.3
Carbon source: oil	49.2	59.3	62.4	56.0	1.37	11.8
S. griseus	42.7	80.0	63.3	90.1	45.21[c]	15.7
B. subtilis						
soyabean extract	142.9[a]	59.5[a]				
peptone+meat extract	145.7[a]	77.8[a]				

[a] 16- and 40-h cultures, resp. [b] $P = 0.05$. [c] $P = 0.01$.

enzyme increased about fivefold during growth of *S. griseus* although the total pool of nicotinamide nucleotides was doubled. This paradox was explained by the fact that the reduced forms, NADH and NADPH, resistant to the action of NAD glycohydrolase, corresponded to about 98% of the total pool of nicotinamide dinucleotides in this strain. As the role of NAD glycohydrolase in regulation of the amount of nicotinamide coenzymes is doubtful in *S. griseus*, it may be significant that the activity of NAD glycohydrolase was 20 times lower in the non-productive mutant of *S. griseus*.

Data given in Tables 3 and 4 refer to the concentration of nicotinamide coenzymes and their respective oxido-reduction state in microorganisms producing peptide antibiotics, in biosynthesis of which NADPH is not directly involved. In *B. subtilis* NAD^+ constituted about 70% of the total pool (Table 3). The decrease of this pool in the stationary phase of growth involved equally all forms of the nucleotides, the NADH/NAD^+ and the NADPH/$NADP^+$ ratios were 0.3 - 0.4 and remained the same during the exponential and stationary phases of growth. Most of the antibiotic was produced in the latter phase. In contrast, in *S. griseus* producing another peptide antibiotic, viomycin, NADH was a predominant form both in the productive and non-productive mutants (Table 4). In the productive mutant at the end of growth the size of the total pool was almost twice as great as in the non-productive one, due to the increased concentration of NADH, resulting also in the higher NADH/NAD^+ ratio. The increase in the NADH/NAD^+ ratio during growth of the highly productive mutant was associated with the corresponding changes in the NADPH/$NADP^+$ ratio. Total content of $NADP^+$ and NADPH did not exceed 2% of the total pool and was constant during growth of this organism. The decrease in concentration of NADP and NADPH in addition to the lack of changes in their oxido-reduction state in *B. subtilis* and accumulation of the reduced coenzymes in *S. griseus* show that biosynthesis of both peptide antibiotics is unrelated to NADPH.

Investigations of the pool of nicotinamide coenzymes in biosynthesis of macrolide antibiotics concerned: non-polyene erythromycin with a saturated polyketide lactone ring synthesized from propionate units (7 C_3) and the polyene tetraene antibiotics with a conjugated double bond system in the lactone ring synthesized from the acetate and propionate units (1 C_2+2 C_3+8 C_2+1 C_3+7 C_2) (Fig. 1).

Table 2

NAD glycohydrolase and the relative content of reduced nicotinamide dinucleotides in Streptomyces griseus *producing viomycin*

The values are averages, ±S.E., of 3 experiments.

Growth	Activity (μmol/mg protein/5 min)	NADH+NADPH (% of total pool)
24	8.4±4.17	98.8
48	27.6±2.87	97.7
72	32.3±5.33	97.4
96	43.8±7.83	98.0

The former antibiotic was produced by *S. erythreus* and the latter by *S. noursei* var. *polifungini*. Studies on *S. erythreus* included the effect of propanol, added in 80 mM concentration to the culture medium; the addition of propanol resulted in an about twofold stimulation of erythromycin production. In *S. noursei* changes in the nucleotide pool were followed in cultures in which lipids and carbohydrates were used alternatively as carbon source.

Concentration of NAD^+ and NADH was increasing to the same extent during growth of *S. erythreus* (Table 5) and the $NADH/NAD^+$ ratio neither depended on

Table 3

Nicotinamide dinucleotides in Bacillus subtilis *producing bacitracin*

The bacteria were grown on the complex soluble medium containing soyabean extract. The values, μg/g wet wt., are averages, ± S.E., of 4 experiments.

Growth (h)	NAD^+	NADH	$NADP^+$	NADPH	NADH/ /NAD^+	NADPH/ /$NADP^+$
16	98.1±6.3	27.5±3.2	11.6±2.1	5.7±0.08	0.28	0.49
40	40.4±3.3	9.9±0.1	6.5±1.4	2.7±0.02	0.25	0.42
L.S.D. *P* 0.05	35.6	11.4	11.4	1.0		

Table 4

Nicotinamide dinucleotides in mutants of Streptomyces griseus *producing viomycin*

The values, μg/g wet wt., are averages, ±S.E., of 3 experiments.

Growth (h)	NAD^+	NADH	$NADP^+$	NADPH	NADH/ /NAD^+	NADPH/ /$NADP^+$
Non-productive mutant						
24	2.3±0.47	49.5± 6.52	0.2±0.09	1.2±0.38	21.6	6.0
48	1.4±0.88	33.9± 9.05	0.6±0.30	2.1±0.42	24.2	3.5
72	1.1±0.32	46.3± 2.60	0.6±0.07	4.0±0.38	42.0	6.7
96	1.1±0.22	44.5± 8.62	0.4±0.16	3.6±0.68	40.5	9.0
Highly-productive mutant						
24	0	47.3± 6.32	0	0.7±0.14	—	—
48	0.3±0.02	76.8± 8.00	0.4±0.04	1.1±0.14	256	2.8
72	0.2±0.01	64.9±10.20	0.1±0.04	1.3±0.22	324	13.0
96	0.1±0.02	87.7± 9.36	0.1±0.04	1.6±0.37	877	16.0

the growth phase nor reflected changes in erythromycin biosynthesis stimulated by the addition of propanol. A distinct decrease was, however, observed in the NADPH/NADP$^+$ ratio in the 48-h cultures, i.e. at the beginning of erythromycin biosynthesis. A still more pronounced, about fourfold decrease was noted in the cultures in which

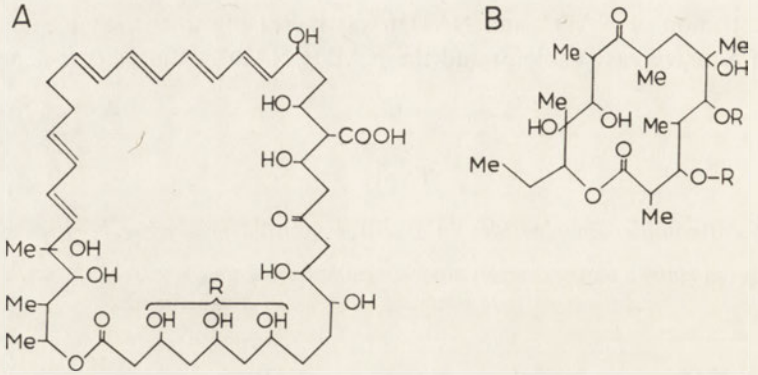

Fig. 1. The lactone ring of macrolide antibiotics: A, polyene: tetraene antibiotics; B, non-polyene: erythromycin A. R, sugar moieties; Me, methyl groups.

biosynthesis of erythromycin was stimulated by propanol. In the course of further biosynthesis up to 96 h of growth concentrations of NADP$^+$ and NADPH were increased to the same extent and no further change in the NADPH/NADP$^+$ ratio took place.

A similar phenomenon was observed in *S. noursei* (Table 6) grown on carbohydrates: the amount of NADP$^+$ and NADPH was increased during growth but the NADPH/NADP$^+$ ratio decreased about threefold in the 48-h cultures, i.e. at the initial stage of antibiotic biosynthesis, and remained unchanged during the stationary phase of growth. In the 24-h cultures of *S. noursei* grown on media in which lipids provided the only carbon source both the NADPH/NADP$^+$ and NADH/NAD$^+$ ratios were about 3 times lower as compared with the cultures grown on carbohydrates, probably due to the inhibition of the Krebs cycle by the free fatty acids or their CoA derivatives (Weber, Lea, Convery & Stamm, 1967) and the repression of NADP-dependent isocitrate and glucose-6-phosphate dehydrogenases in *S. noursei* grown on oil (Roszkowski *et al.*, 1971). The lowered NADPH/NADP$^+$ ratio remained unchanged throughout the 24 - 96 h growth period. The observed differences in the oxido-reduction state of nicotinamide dinucleotide phosphates in *S. noursei* grown on the two media did not affect the yield of the antibiotic. Besides, the ability to synthesize polyene antibiotics in *S. noursei* mutants was not correlated with the activities of NADP-dependent dehydrogenases responsible for regeneration of NADPH (Roszkowski *et al.*, 1971). This suggests that NADPH is not a limiting factor in biosynthesis of these antibiotics.

Table 5

Nicotinamide dinucleotides in Streptomyces erythreus

The values, μg/g wet wt., are averages, ±S.E, of 7 experiments.

Growth (h)	NAD^+	NADH	$NADP^+$	NADPH	NADH/ /NAD^+	NADPH/ /$NADP^+$
Medium non-supplemented with propanol						
24	28.2±4.9	24.0±3.2	0.3±0.04	3.3±0.7	0.9	10.8
48	46.1±6.8	32.0±2.4	1.1±0.4	7.9±1.5	0.7	6.9
72	36.3±6.5	25.0±3.9	1.4±0.4	8.9±1.0	0.7	6.5
96	32.3±3.5	30.1±4.2	1.6±0.4	10.6±1.8	0.9	6.4
Medium supplemented with propanol						
24	26.3±3.0	17.7±4.9	0.1±0.05	3.6±1.8	0.7	36.0
48	28.8±4.8	16.0±4.0	0.7±0.2	6.3±2.9	0.6	8.8
72	25.4±3.1	19.8±5.1	1.1±0.1	10.7±1.6	0.8	9.7
96	24.3±2.5	21.4±5.1	1.3±0.3	10.3±1.6	0.9	8.2
L.S.D. *P* 0.05	10.0	7.8	0.55	2.6		

Table 6

Nicotinamide dinucleotides in Streptomyces noursei *var.* polifungini

The cultures were grown on the carbohydrate or oil as a sole carbon source. The values, μg/g wet wt., are averages, ±S.E., of 6 experiments.

Growth (h)	NAD^+	NADH	$NADP^+$	NADPH	NADH/ /NAD^+	NADPH/ /$NADP^+$
Carbon source: carbohydrates						
24	15.0±3.94	16.5±2.51	0.4±0.09	2.9±0.49	1.1	7.2
48	26.7±6.36	20.1±1.48	1.6±0.28	4.4±1.19	0.8	2.7
72	29.5±6.24	18.8±3.10	1.6±0.41	5.7±0.51	0.6	3.6
96	30.4±6.93	19.6±2.67	2.1±0.54	5.4±0.69	0.6	2.6
120	31.8±6.25	22.6±2.73	2.0±0.39	6.0±0.79	0.7	3.0
L.S.D. *P* 0.05	8.71	5.4	0.77	1.75		
Carbon source: oil						
24	36.2±4.43	9.3±0.42	1.4±0.53	2.3±0.40	0.3	1.6
48	34.2±5.84	17.2±0.87	2.8±0.58	5.1±0.65	0.5	1.8
72	38.1±8.54	15.5±2.50	3.1±0.60	5.7±0.41	0.4	1.8
96	30.4±8.63	17.1±2.89	3.3±0.71	5.0±0.80	0.6	1.5
120	25.7±6.96	20.2±4.98	3.0±0.37	7.4±1.74	0.8	2.5
L.S.D. *P* 0.05	9.1	6.4	0.97	2.33		

Table 7

Concentration of nicotinamide dinucleotide phosphates in Streptomyces

The values are expressed as percentages of the total pool.

Strain and medium	Growth (h)			
	24	48	72	96
S. erythreus				
non-supplemented with PrOH	5.9	10.4	14.4	16.3
supplemented with PrOH	7.8	13.5	20.5	20.3
S. noursei				
Carbon source: carbohydrates	9.5	11.4	13.1	13.1
Carbon source: oil	7.5	13.3	14.1	14.8

The increase in concentration of NADPH during biosynthesis of macrolide antibiotics in *S. erythreus* and *S. noursei* was associated with the increased percentage of nicotinamide dinucleotide phosphates in the total pool of nicotinamide coenzymes. The calculated data presented in Table 7 show that $NADP^+$ and NADPH formed from 6 to 10% of the total pool in the 24-h cultures of *Steptomyces* producing macrolide antibiotics, and their participation in this pool increased to 13 - 20% in the 96-h cultures.

The presented results indicate that NADPH deficiency can not be a signal for the start of biosynthesis of macrolide antibiotics as it was postulated by Behal, Cudlin & Vanek (1969) for biosynthesis of tetracycline, another acetate derived antibiotic. Yalpani, Willecke & Lynen (1969) observed formation of triacetic lactone from acetyl- and malonyl-CoA by the baker's yeast fatty acid synthetase when NADPH was not available for reduction. The authors from the same laboratory (Dimroth, Walter & Lynen, 1970) proved, however, the existence of two separate multienzyme synthetase systems in *Penicillium patulum:* one synthesizing fatty acids and the other responsible for the synthesis of 5-methylsalicylic acid from acetyl- and malonyl--CoA. Formation of the polyacyl antibiotics from acetate and propionate units may result therefore from the induction of the new synthetase system having different affinity to NADPH. The appearance in the stationary phase of growth of the enzyme responsible for biosynthesis of antibiotics is exemplified by the induction of the ornithine-activating enzyme in *Bacillus brevis* synthesizing gramicidin S (Otani, Jamanoi & Saito, 1969) or the system responsible for the formation of phenoxazinone in biosynthesis of actinomycin (Marshall, Redfield, Katz & Weissbach, 1968).

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DWUNUKLEOTYDY NIKOTYNAMIDOWE W BIOSYNTEZIE ANTYBIOTYKÓW PEPTYDOWYCH I MAKROLIDOWYCH

Streszczenie

1. Na udział NADPH w syntezie antybiotyków makrolidowych wskazuje gwałtowny spadek stosunku NADPH/$NADP^+$ z chwilą rozpoczęcia syntezy erytromycyny przez *S. erythreus* lub syntezy polienowych antybiotyków tetraenowych przez *S. noursei*.

2. Podczas biosyntezy peptydowego antybiotyku wiomycyny u *S. griseus* zredukowane formy obu nukleotydów ulegają nagromadzeniu, natomiast ich stan oksydoredukcyjny nie ulega żadnym zmianom u *B. subtilis* syntetyzującego inny antybiotyk peptydowy — bacytracynę.

3. Bardzo wysokiej czynności NAD glikohydrolazy u *S. griseus*, przewyższającej czynność tego enzymu u *Neurospora crassa*, towarzyszy przewaga (97%) form zredukowanych w puli nukleotydów nikotynamidowych. U pozostałych badanych szczepów czynność tego enzymu jest o cztery rzędy wielkości niższa.

Received 8 January, 1971.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

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CYTOSINE AND CYTIDINE ANALOGUES: SYNTHESIS AND PROPERTIES OF 5-ETHYLCYTOSINE, 5-ETHYLCYTIDINE AND A NUMBER OF THEIR DERIVATIVES

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1) The preparation and properties of 2,4-diethoxy-5-ethylpyrimidine and a series of its derivatives is described. 2) These were employed for the synthesis of a number of model compounds, such as 5-ethyl-1-methylcytosine and some alkylamino derivatives, the properties of which are described. 3) N^4,N^4,5-trimethylcytosine and 1,N^4,N^4,5-tetramethylcytosine have also been synthesized. 4) A new procedure is outlined for the synthesis of 5-ethylcytosine in good yield, with 5-ethylisocytosine as a by-product. 5) The introduction of a 5-ethyl substituent into 2,4-dichloropyrimidine appreciably facilitates nucleophilic attack of the 4-position by ammonia. 6) The Hilbert-Johnson rearrangement has been applied to the synthesis of 5-ethylcytidine and some of its N^4-alkyl derivatives in good yield; an advantage of this pathway to 5-ethylcytidine is that it permits of the simultaneous preparation of 5-ethyluridine in equally good yield. 7) The ultraviolet absorption spectra and pK_a values of all the foregoing compounds are described and discussed. 8) Reference is made to the potential biological significance of the 5-ethyl cytosine and cytidine analogues.

The following report, devoted to a description of the synthesis of 5-ethylcytosine, its ribonucleosides, and a number of analogues and intermediates, appeared desirable from several different points of view, not the least of which is the fact that 5-ethylcytosine is a base analogue of both cytosine and 5-methylcytosine, all of which should exhibit similar base-pairing properties.

It has already been shown that 5-ethyluracil can replace thymine in bacterial (Piechowska & Shugar, 1965) and bacteriophage (Pietrzykowska & Shugar, 1966, 1967) DNA. Furthermore 5-ethyldeoxyuridine exhibits anti-viral activity (Świerkowski & Shugar, 1969) and its incorporation into phage DNA is, as might be anticipated, non-mutagenic (Shugar, Świerkowski, Fikus & Barszcz, 1967; Świerkowski & Shugar, 1969). It has also been shown that L-ethionine induced hepatic carcinoma is accompanied by extensive ethylation of nucleic acids, in particular

tRNA (Ortwert & Novelli, 1969), although by a different metabolic pathway than methylation.

Replacement of uracil residues in poly(rU)[1] by thymine, or of cytosine residues in poly(rC) by 5-methylcytosine, leads to a pronounced enhancement of the thermal stability of helical structures of the resulting poly(5MeU) and poly(5MeC) (Shugar & Szer, 1962; Szer, Świerkowski & Shugar, 1963; Szer & Shugar, 1966a). However, when the thymine residues in poly(5MeU) are replaced by 5-ethyluracil, the helical structures formed by the resulting poly(5EtU) exhibit a much lower stability. While there is as yet no theoretical interpretation of these findings, it is of interest that the reduced stability of helical polynucleotides containing 5-ethyluracil residues, as compared to 5-methyl, is reflected also in the behaviour of natural nucleic acids containing incorporated 5-ethyluracil (Pietrzykowska & Shugar, 1967). It is consequently of obvious interest to prepare and examine the properties of poly(5EtC) as compared to poly(5MeC) and poly(C).

The foregoing obviously requires the preparation of the riboside of 5-ethylcytosine and its pyrophosphorylation to give 5-ethylcytidine-5′-diphosphate (5EtCDP), a potential substrate for polynucleotide phosphorylase. The preparation of 5EtCDP and poly(5EtC) and the properties of the latter will be described elsewhere. The present study is limited to the synthesis of the base, and the ribonucleosides, and a variety of analogues of these including, in particular, new data on the preparation and properties of 5-substituted cytosine derivatives containing an exocyclic dialkylamino substituent, which an earlier investigation (Kulikowski, Żmudzka & Shugar, 1969) had shown to be difficult of achievement, for reasons fully discussed therein. Furthermore the procedures developed here for the synthesis of 5-ethylcytosine glycosides were such as to permit of the simultaneous preparation of the now known 5-ethyluracil glycosides by another method.

Synthesis of 5-ethylpyrimidine derivatives

The synthesis of 5-ethylcytosine, its 1-methyl derivative and 1-β-D-riboside, as well as the corresponding exocyclic alkylamino analogues, called for the preparation of an appropriate derivative of 5-ethylpyrimidine, e.g. some derivative of the well-known 2,4-dialkoxypyrimidine (Hilbert & Johnson, 1930a; Schmidt-Nickels & Johnson, 1930) or the more recently described bis-(trimethylsilyl)cytosine (Nishimura & Iwai, 1964). Both of these readily undergo Hilbert-Johnson rearrangement either with methyl iodide or with an appropriately blocked halogeno sugar (Hilbert & Johnson, 1930a; Sakai, Pogolotti & Santi, 1968; Hilbert & Johnson, 1930b; Nishimura, Shimizu & Iwai, 1964) to provide the corresponding 1-methyl or 1-glycoside derivatives. Our desire to prepare the exocyclic alkylamino derivatives of necessity led to the choice of a 2,4-dialkoxypyrimidine as starting compound, i.e. 2,4-dialkoxy-5-ethylpyrimidine. The enhanced resistance to rearrangement at elevated tempera-

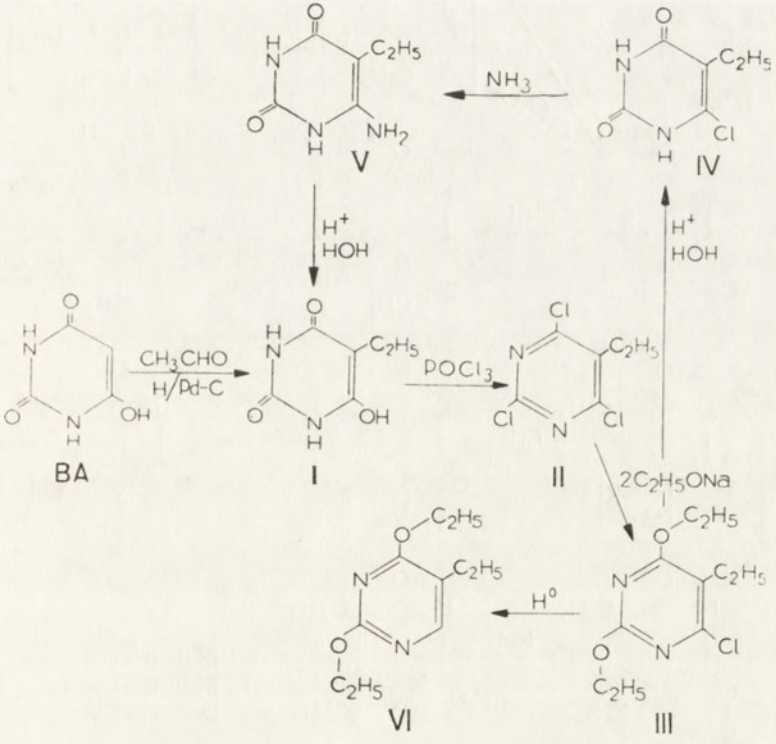
[1] Abbreviations for polynucleotides used in this text are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, 1969 (*Europ. J. Biochem.* **15**, 203, 1970).

tures of ethoxypyrimidines, as compared to methoxypyrimidines (Schmidt-Nickels & Johnson, 1930; Brown & Foster, 1965), led to the selection of 2,4-diethoxy-5--ethylpyrimidine.

Synthesis of 2,4-diethoxy-5-ethylpyrimidine and its derivatives

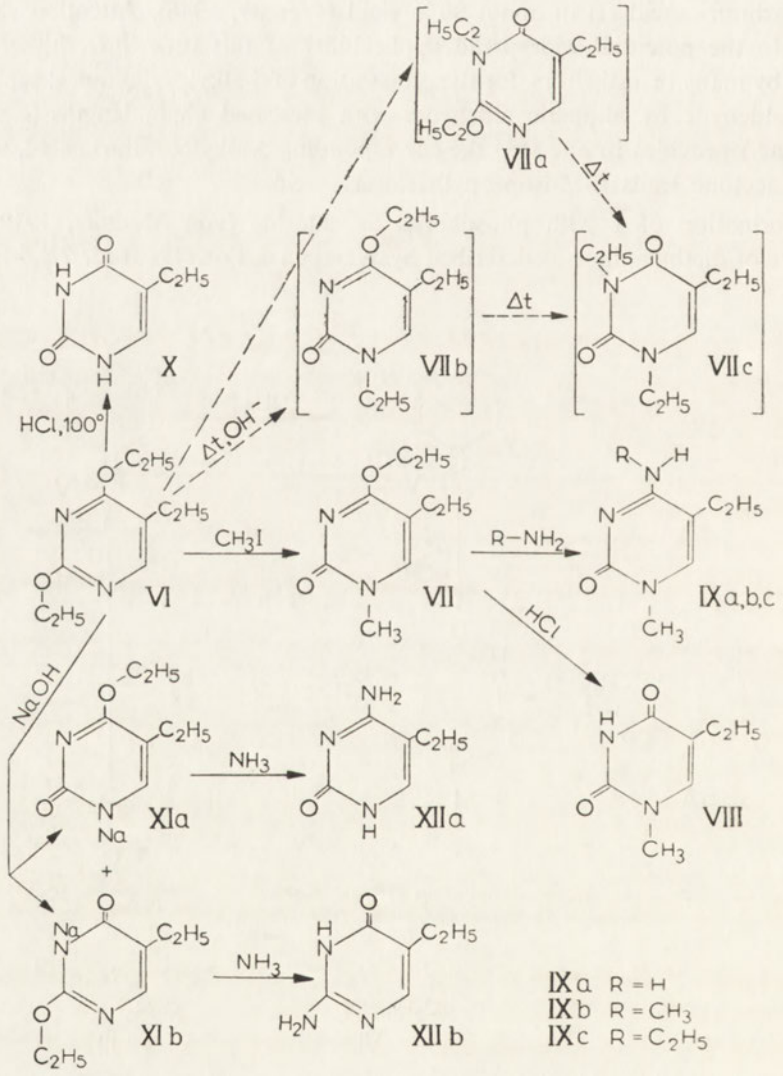
2,4-Diethoxy-5-ethylpyrimidine (VI) is accessible *via* 5-ethylbarbituric acid (I, 4-stage synthesis, see Scheme 1) or by a principal synthesis *via* 2-thio-5-ethyluracil (5-stage synthesis, see Scheme 4). The former, apparently shorter, procedure was adopted. Barbituric acid (BA, Scheme 1) was subjected to catalytic hydrogenation at elevated pressure and temperature in the presence of acetaldehyde to yield 5--ethylbarbituric acid (I) in about 90% yield (Wenner, 1946). Attention should be drawn to the potential widespread applicability of this procedure, hitherto overlooked by many investigators, for the preparation of 5-alkylpyrimidines: replacement of acetaldehyde by aliphatic aldehydes with increased chain lengths (e.g. propyl, butyl, etc.) provides in one step the corresponding 5-alkylbarbituric acid, while the use of acetone leads to 5-isopropylbarbituric acid.

Chlorination of I with phosphorus oxychloride (von Merkatz, 1919) in the presence of diethylaniline as described by Wempen & Fox (1963) for 2,4,6-trichloro-



Scheme 1. Synthesis of 2,4-diethoxy-5-ethylpyrimidine.

pyrimidine, gave 2,4,6-trichloro-5-ethylpyrimidine (II) in 75% yield. Treatment of II with 2 molar equivalents of sodium ethoxylate at room temperature led to 6--chloro-2,4-diethoxy-5-ethylpyrimidine (III) in 60% yield. This compound exhibited a UV absorption spectrum identical with that for the previously reported 6-chloro--2,4-dimethoxy-5-ethylpyrimidine (Shapira, 1962), and readily underwent hydrolysis in acid to 6-chloro-5-ethyluracil (IV) which, in turn, was readily aminated to 6--amino-5-ethyluracil (V). The latter, on heating in 1 N-HCl, deaminated to give 5-ethylbarbituric acid (I). The UV spectra of III, IV and V closely resembled those reported for the analogous 6-chloro-2,4-diethoxypyrimidine (Cresswell & Wood, 1960), 6-chlorouracil (Wempen & Fox, 1964), and 6-aminouracil (Fink, Adams



Scheme 2. Synthesis of derivatives of 2,4-diethoxy-5-ethylpyrimidine.

& Pfleiderer, 1964), due allowance being made for the bathochromic shift due to the 5-ethyl substituent.

Reduction of III (Scheme 1) gave a chlorine-free oil, identified as 2,4-diethoxy--5-ethylpyrimidine (VI). Treatment of VI with boiling HCl (Scheme 2) gave the known 5-ethyluracil (X), while hydrolysis in NaOH led to a mixture of the sodium salts of 4-ethoxy-5-ethyl-2(1H)-pyrimidinone (XIa) and 2-ethoxy-5-ethyl-4(3H)-pyrimidinone (XIb). On treatment with methyl iodide, VI underwent a Hilbert-Johnson rearrangement to 4-ethoxy-5-ethyl-1-methyl-2(1H)-pyrimidinone (VII). Acid hydrolysis of VII yielded 5-ethyl-1-methyluracil (VIII), while treatment at elevated temperature with alcoholic ammonia or primary amines led to 5-ethyl-1-methylcytosine (IXa) and its alkylamino analogues (IXb,c, see also below).

The UV spectrum of redistilled VI (referred to below as obtained by Method A) exhibited an absorption maximum (λ_{max}^{pH7} 264 nm) close to that for 2,4-diethoxy-5--methylpyrimidine (λ_{max}^{pH7} 265 nm) (Wittenburg, 1966), but shifted bathochromically by 2 nm with respect to VI obtained by ethoxylation of 2,4-dichloro-5-ethylpyrimidine (Method B, see below). However, elementary analysis of VI gave the correct formula, $C_{10}H_{16}N_2O_2$, thus excluding contamination by hydrolysis products, and suggesting that the products responsible for the bathochromic shift in the spectrum of VI arise from isomerization of the latter, i.e. 1,5-diethyl-4-ethoxy-2(1H)-pyrimidinone (VIIb), 2-ethoxy-3,5-diethyl-4(3H)-pyrimidinone (VIIa) and 1,3,5-triethyluracil (VIIc). Gas chromatography of VI exhibited the presence of two minor contaminants in proportions of approximately 2% and 8%. Resort was then had to thin-layer chromatography of VI on silica gel; development with ethyl acetate - methanol, 97:3 v/v (see Table 3) pointed to the slower migrating impurity (2%) as VIIb, and the faster migrating impurity (8%) as VIIa or VIIc. Such isomers are known to result from thermal rearrangement of 2,4-dialkoxypyrimidines in the presence of base (Schmidt--Nickels & Johnson, 1930; Brown & Foster, 1965). It is therefore most likely that they arose in this case during the fractional vacuum distillation of VI in the presence of traces of residual base (KOH).

Synthesis of 5-ethyl-1-methylcytosine and its N^4*-alkyl analogues*

The preparation of 5-ethyl-1-methylcytosine (IXa) and its N^4-methyl (IXb) and N^4-ethyl (IXc) derivatives (Scheme 2), which are structural analogues of 5-ethylcytosine nucleosides, made use of VII which, on treatment at elevated temperature with anhydrous alcoholic solutions of ammonia, methylamine, and ethylamine, was converted in good yield to 5-ethyl-1-methylcytosine (IXa), 1,N^4-dimethyl-5--ethylcytosine (IXb) and N^4,5-diethyl-1-methylcytosine (IXc), respectively. By contrast, treatment of VII with dimethylamine, even at higher temperatures or for longer periods of time, resulted in formation only of 5-ethyl-1-methyluracil, a finding similar to that observed in attempts to dialkylaminate 1,5-dimethyl-4-ethoxy-2(1H)--pyrimidinone (see Kulikowski *et al.*, 1969, for discussion of these results).

It has also been shown that dimethylamination of 1,5-dimethyl-4-thiothymine

also does not occur (Kulikowski *et al.*, 1969). Since it was desirable to have a suitable reference compound for comparison purposes with the previously obtained N^4,N^4,5--trimethyl-2′-deoxycytidine (Kulikowski *et al.*, 1969), attempts were now directed towards the obtention of 1,N^4,N^4,5-tetramethylcytosine by dimethylamination of the corresponding 4-chloropyrimidine. Thymine (XVIII, see Scheme 3) was therefore chlorinated with phosphorus oxychloride to obtain 2,4-dichloro-5-methylpyrimidine (XIX); treatment of the latter with aqueous dimethylamine (Koppel, Springer, Robins & Cheng, 1962) gave 2-chloro-4-dimethylamino-5-methylpyrimidine (XX) which, with concentrated sodium ethoxylate, led to 2-ethoxy-4-dimethylamino-5--methylpyrimidine (XXI) in the form of an oil. The UV spectrum of XXI was similar to that for 2-ethoxy-4-aminopyrimidine (Shugar & Fox, 1952), allowance being made for a bathochromic shift and a decrease in the ratio $\varepsilon_{max}^{pH1}/\varepsilon_{max}^{pH7}$ (Table 2). Heating of XXI in conc. HCl to 100°C led to N^4,N^4,5-trimethylcytosine (XXIII). Treatment of XXI with methyl iodide led to the tertiary compound 2-ethoxy-4--dimethylamino-5-methylpyrimidine methiodide (XXIIa) which, on heating with a solution of Ag_2SO_4 gave the sought-for 1,N^4,N^4,5-tetramethylcytosine (XXIIb). The UV spectrum of XXIIb resembled that of the known 1,N^4,N^4-trimethylcytosine (Szer & Shugar, 1966b), allowance being made for the bathochromic shift due to the 5-methyl substituent; and was very similar to the spectrum of N^4,N^4,5-trimethyl--2′-deoxycytidine (Kulikowski *et al.*, 1969).

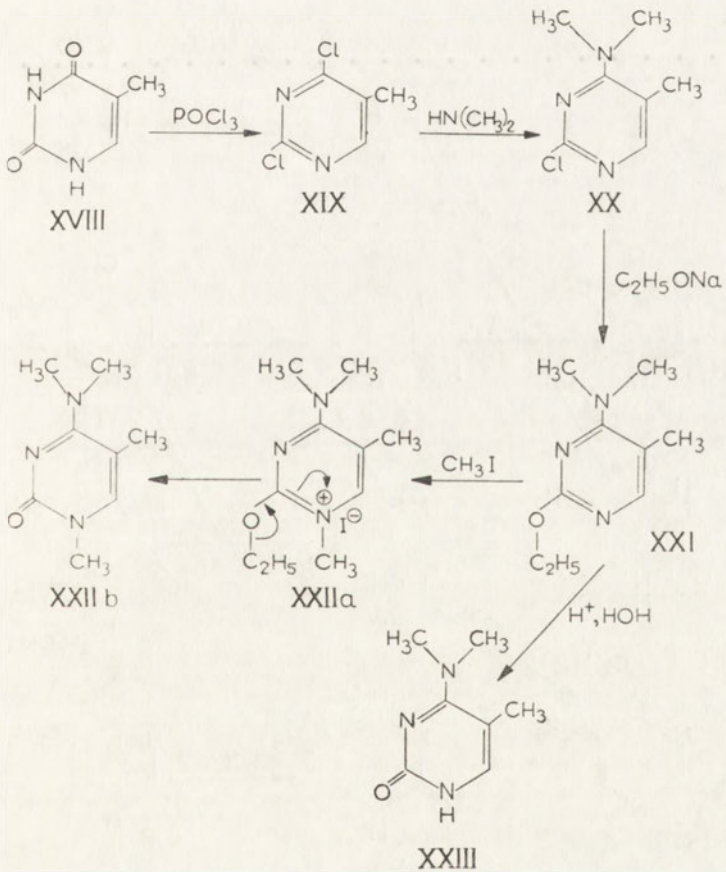
Synthesis of 5-ethylcytosine and 5-ethylisocytosine

5-Ethylcytosine (XIIa), first reported by Johnson & Menge (1906), has since then gone unnoticed notwithstanding that it is a close analogue of 5-methylcytosine which occurs in natural nucleic acids. The synthesis of this compound by Johnson & Menge (1906), was based on the reaction pathway for 5-methylcytosine (Wheeler & Johnson, 1903, 1904); no yields were given for the last two stages, but it has been noted by Hitchings, Elion, Falco & Russell (1949) that removal of the 2-thiol group in the presence of a 4-amino substituent is not efficient. Application of the Hilbert--Johnson (1930c) procedure for cytosine also did not appear promising: in effect, amination of 2,4-dichloropyrimidine leads to a mixture of 2-chloro-4-amino- and 2-amino-4-chloropyrimidines, which may be separated readily only after transformation to alkoxypyrimidines (Hilbert, 1934). The proportion of isomers in this case is about 2:3, thus leading to a low yield of the desired 4-amino isomer. There are, on the other hand, reports that amination of 2,4-dichloro-5,6-dimethylpyrimidine at 100°C (Schlenker, 1901) and 2,4-dichloro-6-methylpyrimidine at 90°C (Marshall & Walker, 1951) provide mixtures of aminochloropyrimidines which may be fractionated in good yield. In both these instances the ratio of the two isomers was slightly in favour of the 4-amino-2-chloro, leading to the suggestion that substitution of the 5- and/or 6-positions favours attack of the 4-position by amines as a result of partial localization of double bonds in the pyrimidine rings.

A second possibility would be the alkaline hydrolysis of the above-mentioned 2,4-diethoxy-5-ethylpyrimidine (VI), which should lead to a mixture of the sodium

salts of the 2- and 4-ethoxy derivatives (Hilbert & Jansen, 1935). Since the amount of VI at our disposal was substantial, this procedure was initially followed (see Scheme 2). Alkaline hydrolysis of VI was carried out as described by Szer & Shugar (1968) to give the sodium salts of 4-ethoxy-5-ethyl-2(1H)-pyrimidinone (XIa) and 2-ethoxy-5-ethyl-4(3H)-pyrimidinone (XIb). Amination of each of these at elevated temperature gave the required 5-ethylcytosine (XIIa), and 5-ethylisocytosine (XIIb).

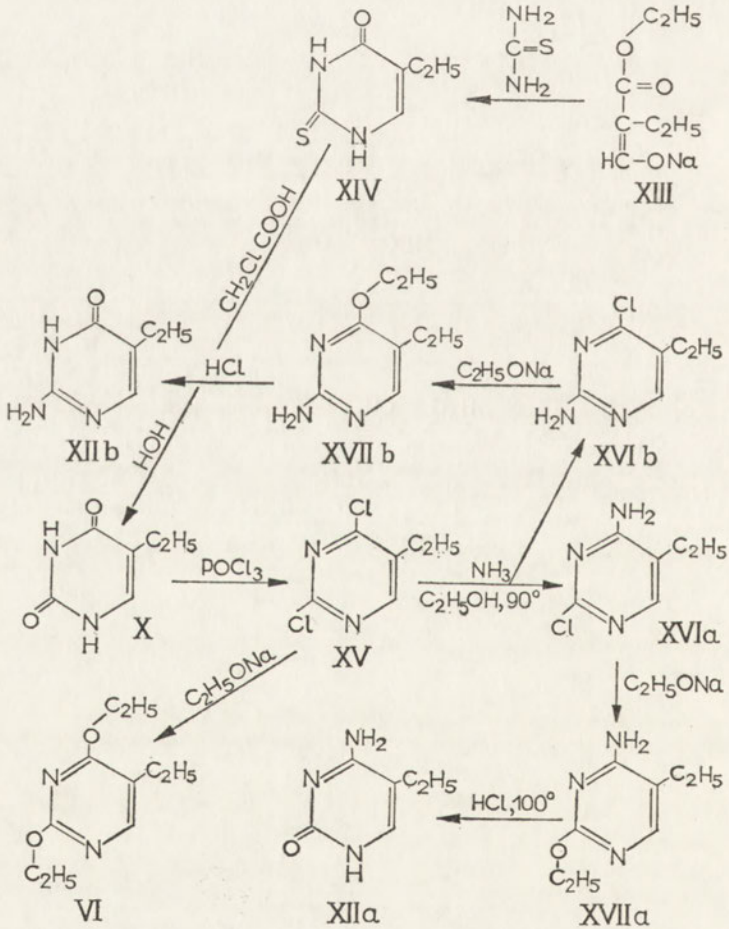
However, the foregoing procedure provided XIIa in only 17% yield (with respect to the starting compound VI). A more efficient method was consequently undertaken, based on the first of the above variants (Scheme 4). 5-Ethyl-2-thiouracil (XIV), obtained by a modification of the procedure of Burckhalter & Scarborough (1955), was hydrolysed in chloroacetic acid to give 5-ethyluracil (X), which was chlorinated with phosphorus oxychloride to 2,4-dichloro-5-ethylpyrimidine (XV). Treatment of XV with an excess of sodium ethoxylate led to 2,4-diethoxy-5-ethylpyrimidine (VI, Method B) which, following distillation, was much purer than that obtained by another procedure (Method A, see above), spectrally and chromatograph-



Scheme 3. Synthesis of N^4,N^4-dimethyl derivatives of 5-methylcytosine and 1,5-dimethylcytosine.

ically. It reacted with methyl iodide to give VII in good yield. Following a search for optimal amination conditions of XV, it was found that ethanolic ammonia at 90°C gave an 80% yield of crystalline amination products with a 10:1 ratio of the desired 4-amino isomer to the 2-amino. The final reaction mixture contained no starting compound, nor the 2,4-diamino derivative. Furthermore, the 2-amino isomer (XVIb) proved practically insoluble in cold ethanol and was easily filtered off, following which the filtrate was brought to dryness and the resulting residue of 4-amino-2-chloro-5-ethylpyrimidine (XVIa), following crystallization from dilute *n*-hexane or *n*-heptane solutions, required no further purification.

Ethoxylation of XVIa and XVIb at elevated temperature yielded the corresponding 4-amino-2-ethoxy-5-ethylpyrimidine (XVIIa) and 2-amino-4-ethoxy-5-ethylpyrimidine (XVIIb). These, following removal of NaCl by filtration, were employed for the following steps without further purification. Hydrolysis with conc. HCl at 100°C gave 5-ethylcytosine (XIIa) and 5-ethylisocytosine (XIIb) in good yield. Both of these exhibited UV spectra similar to those for 5-methylcytosine and iso-



Scheme 4. Synthesis of 5-ethylcytosine and 5-ethylisocytosine from 5-ethyl-2-thiouracil.

cytosine (Table 2), migrated faster than the latter on silica gel and on cellulose (Table 3), and exhibited pK_a values for protonation of the ring N-3 nitrogens slightly higher than those for the 5-methyl derivatives. The overall yield for XIIa was 50%, and for XIIb 4% (with respect to 5-ethyluracil).

Synthesis of 1-β-D-ribofuranosides of 5-ethyluracil and 5-ethylcytosine

Preparation of 5-ethyluridine (XXIII, 1-β-D-ribofuranosyl-5-ethyluracil) has previously been reported by several authors (Shapira, 1962; Świerkowski & Shugar, 1969; Muraoka, Takada & Ueda, 1970), all of whom employed the well-known "mercury" method (Fox, Yung, Davoll & Brown, 1956) for the synthesis of this analogue of 5-methyluridine, which is found in tRNA (Littlefield & Dunn, 1958). We were prompted to undertake the synthesis of the desired 5-ethylcytidine *via* the Hilbert-Johnson rearrangement, since the same pathway could then be used simultaneously for obtaining 5-ethyluridine.

Attempts to obtain 5-methyluridine in this way have been reported to give varying results, depending on the reaction conditions employed. Reaction of 2,4--diethoxy-5-methylpyrimidine with acetylbromoribose (Visser, Goodman & Dittmer, 1948) according to the procedure of Howard, Lythgoe & Todd (1947) at 50°C in the absence of solvent eventually gave 1-D-ribosylthymine (configuration not established) in 8% yield, m.p. 252°C, $[\alpha]^D$ 110°. Roberts & Visser (1952) subsequently slightly modified this procedure, using a reaction temperature of 60°C to obtain

Table 1

Apparent pK_a *values (±0.05* pH *units) at 20°C for 5-ethylcytosine, its 1-methyl and 1-β-D-ribosyl derivative, and the corresponding alkylamino analogues*

Compound	pK_{a1}[a]	pK_{a2}[b]
5-Methylcytosine	4.60[c]	12.40[c]
N^4,N^4,5-Trimethylcytosine (XXIII)	4.3	—
1,5-Dimethylcytosine	4.76	—
1,N^4,5-Trimethylcytosine	4.57	—
1,5-Dimethyl-N^4-ethylcytosine	4.58	—
1,N^4,N^4,5-Tetramethylcytosine (XXIIb)	4.48	—
5-Ethylcytosine (XIIa)	4.81	~12.8
5-Ethyl-1-methylcytosine (IXa)	4.95	—
5-Ethyl-1,N^4-dimethylcytosine (IXb)	4.66	—
N^4,5-Diethyl-1-methylcytosine (IXc)	4.70	—
5-Methylcytidine	4.28[d]	~13[d]
5-Ethylcytidine (XXVIIIa)	4.42	~13
5-Ethyl-N^4-methylcytidine (XXVIIIb)	4.14	~13
N^4,5-Diethylcytidine (XXVIIIc)	4.16	~13

[a] For protonation of ring N-3 nitrogen.
[b] For dissociation of carbohydrate hydroxyl(s).
[c] Data from Shugar & Fox (1952).
[d] Data from Fox *et al.* (1959).

the nucleoside in 10% yield, m.p. 175-177°C (configuration not established). Farkaš, Kaplan & Fox (1964) then examined this reaction, with benzene as solvent, and obtained two products in 45% overall yield: one, which readily crystallized, m.p. 175-176°C, $[\alpha]^D$ 50°, to which they ascribed the configuration α, and a second with m.p. 184°C, $[\alpha]^D$ 10°, assigned the configuration β. The ratio of the two anomers was 1:1.

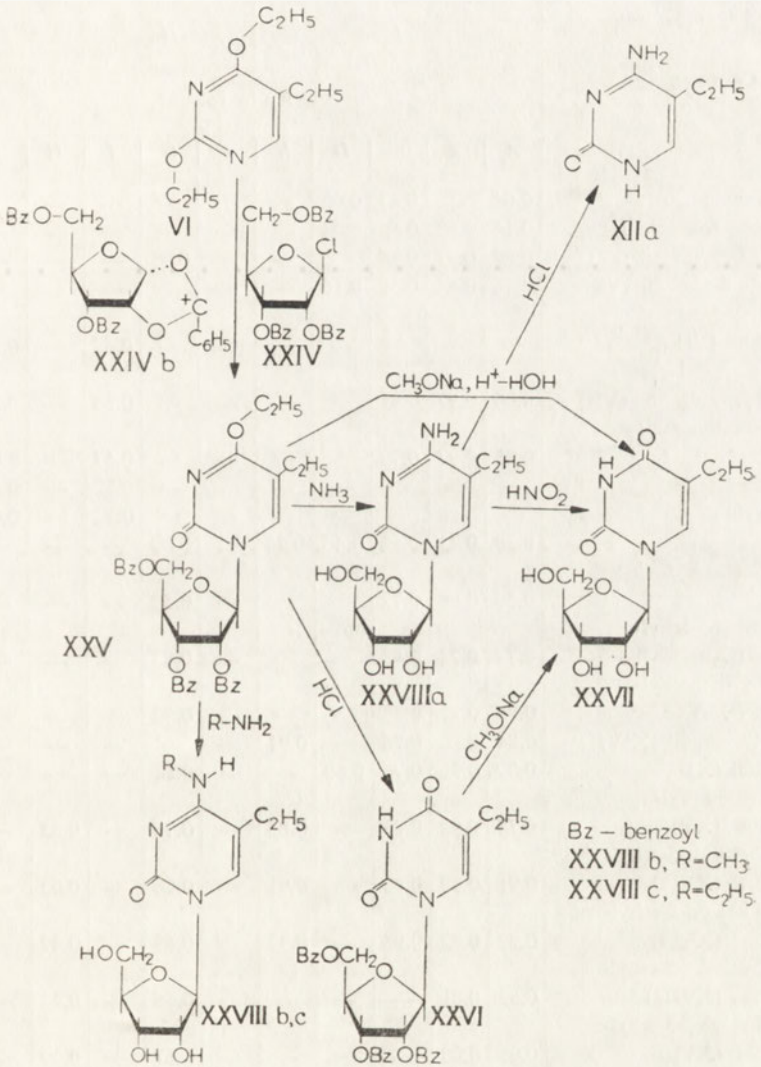
Table 2

Spectrophotometric data for 5-ethylcytosine, its 1-methyl and 1-β-D-ribosyl derivative, and the corresponding alkylamino analogues

Compound	pH	λ_{max} (nm)	ε_{max} ($\times 10^{-3}$)	λ_{min} (nm)	ε_{min} ($\times 10^{-3}$)	$\frac{\varepsilon_{max}^{pH1}}{\varepsilon_{max}^{pH7}}$
5-Methylcytosine[a]	1	283.5	9.79	242	0.9	1.571
	7	273.5	6.23	248	4.3	
	14	289.5	8.05	253.5	1.35	
N^4,N^4,5-Trimethylcytosine (XXIII)	1	298	11.09	252	1.35	1.134
	7	285	9.78	247	6.41	
	14	300	8.73	265	1.55	
1,5-Dimethylcytosine	1	291	11.41	245	0.63	1.480
	7	280	7.71	252	3.19	
1,N^4,5-Trimethylcytosine	1	290	13.19	245	1.33	1.349
	7	278	9.78	252	4.83	
1,5-Dimethyl-N^4-ethylcytosine	1	291	13.42	248	1.31	1.281
	7	278	10.48	253	5.36	
1,N^4,N^4,5-Tetramethylcytosine (XXIIb)	1	305	12.61	255	1.13	1.241
	7	290	10.16	255	5.04	
5-Ethylcytosine (XIIa)	1	283.5	9.46	242	0.93	1.556
	7	274	6.08	252	3.50	
	14	289	7.80	255	1.33	
5-Ethyl-1-methylcytosine (IXa)	1	291	11.43	235	0.93	1.481
	7	280	7.72	253	3.56	
5-Ethyl-1,N^4-dimethylcytosine (IXb)	1	290	12.89	247	1.27	1.322
	7	280	9.75	252	5.01	
N^4,5-Diethyl-1-methylcytosine (IXc)	1	291	10.13	250	0.90	1.300
	7	278	7.79	253	3.99	
5-Methylcytidine[b]	1	288	12.6	245	0.74	1.416
	7	278	8.9	255	5.8	
5-Ethylcytidine (XXVIIIa)	1	288.5	12.35	245.5	0.85	1.411
	7	277.5	8.75	256	5.33	
5-Ethyl-N^4-methylcytidine (XXVIIIb)	1	287	14.26	246	1.44	1.270
	7	275	11.40	251.5	7.7	
N^4,5-Diethylcytidine (XXVIIIc)	1	288	13.83	247	1.32	1.255
	7	276	11.02	252	7.74	

[a] Shugar & Fox (1952).
[b] Fox *et al.* (1959).

A major improvement both in the yield and stereochemical course of this reaction was obtained by the use as solvent of acetonitrile in the presence of molecular sieves (Prystaš & Šorm, 1965, 1966a,b). Under these conditions the reaction of 2,4-dimethoxy-5-methylpyrimidine with 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl chloride (XXIV) (Scheme 5) gave exclusively the β-anomer, m.p. 183 - 184°C, in 67% yield. It was proposed that, in a polar, nucleophilic solvent such as acetonitrile, the acylated halogeno sugar exists principally in the β configuration and, through solvatation, forms a stable cationic orthoester (XXIVb) which can give rise only to the β-anomer (Prystaš & Šorm, 1966a; Pliml & Prystaš, 1967). There exist departures from this rule, e.g. 5-benzyloxymethyl-2,4-dimethoxypyrimidine reacts with protected halo-



Scheme 5. Synthesis of 1-β-D-ribofuranosides of 5-ethyluracil and 5-ethylcytosine.

Table 3

R_F *values of 5-ethylcytosine and 5-ethylcytidine and a number of their derivatives and intermediates*

Ascending chromatography on: Whatman no. 1 paper (*I*), silica gel TLC plates (*II*), and neutral alumina TLC plates (*III*) of 5-ethylpyrimidine derivatives, 1-β-D-ribofuranosides of 5-ethyluracil and 5-ethylcytosine, and a number of their structural analogues and derivatives, with the following solvent systems (ratios in all cases v/v): *A*: water-saturated butanol; *B*: isopropanol - water - conc. HCl (d = 1.18), 65:18.4:16.6; *C*: isopropanol - conc. NH_4OH - water, 7:1:2; *D*: upper phase from a mixture of benzene - ethanol - water, 170:45:10; *E*: upper phase from a mixture at 20°C of *sec.*-butyl alcohol and water; *F*: chloroform - methanol, 70:30; *G*: chloroform - methanol, 85:15; *H*: ethyl acetate; *I*: ethyl acetate - methanol, 97:3; *J*: benzene - ethyl acetate, 8:2.

Compound	*I*					*II*			*III*		
	R_F in solvent										
	A	*B*	*C*	*D*	*E*	*F*	*G*	*I*	*H*	*I*	*J*
Barbituric acid	0.04	—	0.47	0.00	—	—	—	—	—	—	—
5-Ethylbarbituric acid (I)	0.14	0.82	0.62	0.04	—	—	—	—	—	—	—
6-Chloro-5-ethyluracil (IV)	0.88	0.97	0.86	—	—	—	0.93	—	—	—	—
6-Amino-5-ethyluracil (V)	0.66	0.84	0.58	0.16	—	—	—	—	—	—	—
2,4-Diethoxy-5-ethyl-pyrimidine (VI)	—	—	—	—	—	—	—	0.66	—	0.95	0.92
4-Ethoxy-5-ethyl-1-methyl--2(1H)-pyrimidinone (VII)	0.94	0.97	0.91	—	—	—	—	0.30	—	0.54	0.08
5-Ethyl-1-methyluracil (VIII)	0.81	0.97	0.79	—	—	—	—	0.47	—	0.03	0.00
VIIb	—	—	—	—	—	—	—	0.32	—	0.46	—
VIIa or VIIc	—	—	—	—	—	—	—	0.52	—	0.63	—
Cytosine	0.28	0.41	0.54	0.46	0.45	—	0.09	—	—	—	—
5-Ethyl-1-methylcytosine (IXa)	0.59	0.69	0.72	—	—	—	0.58	—	—	—	—
1,N^4-Dimethyl-5-ethyl-cytosine (IXb)	0.77	0.74	0.83	—	—	—	0.83	—	—	—	—
N^4,5-Diethyl-1-methyl-cytosine (IXc)	0.86	0.81	0.85	—	—	—	0.92	—	—	—	—
5-Ethyl-2-thiouracil (XIV)	0.84	0.96	0.76	—	0.91	—	—	—	—	—	—
5-Ethyluracil (X)	0.80	0.92	0.76	0.88	—	—	0.84	—	—	—	—
2,4-Dichloro-5-ethyl-pyrimidine (XV)	0.74	0.92	0.83	—	0.85	—	0.76	—	0.85	—	—
4-Amino-2-chloro-5-ethyl-pyrimidine (XVIa)	0.91	0.80	0.93	—	0.91	—	0.66	—	0.63	—	—
2-Amino-4-chloro-5-ethyl-pyrimidine (XVIb)	0.92	0.82	0.95	—	0.92	—	0.68	—	0.47	—	—
4-Amino-2-ethoxy-5-ethyl-pyrimidine (XVIIa)	0.83	0.81	—	—	—	—	0.66	—	0.44	—	—
2-Amino-4-ethoxy-5-ethyl-pyrimidine (XVIIb)	0.95	0.98	—	—	—	—	0.67	—	0.39	—	—
5-Ethylcytosine (XIIa)	0.51	0.64	0.70	—	—	0.42	0.26	—	—	—	—
5-Ethylisocytosine (XIIb)	0.77	0.78	0.75	—	—	—	0.69	—	—	—	—

Compound	*I*					*II*			*III*		
	R_F in solvent										
	A	*B*	*C*	*D*	*E*	*F*	*G*	*I*	*H*	*I*	*J*
1-(2,3,5-Tri-*O*-benzoyl-β-D--ribofuranosyl)-4-ethoxy--5-ethyl-2(1H)-pyrimidinone (XXV)	—	—	—	—	—	0.95	—	0.66	—	—	0.71
2′,3′,5′-Tri-*O*-benzoyl-5--ethyluridine (XXVI)	—	—	—	—	—	0.82	—	0.67	—	—	0.00
5-Ethyluridine (XXVII)	0.55	0.82	0.74	—	0.76	—	0.31	—	—	—	—
5-Methylcytidine	0.21	0.49	0.58	—	0.41	—	0.06	—	—	—	—
5-Ethylcytidine (XXVIIIa)	0.32	0.58	0.67	—	0.51	—	0.09	—	—	—	—
5-Ethyl-N^4-methylcytidine (XXVIIIb)	0.47	0.62	0.80	—	0.63	—	0.26	—	—	—	—
N^4,5-Diethylcytidine (XXVIIIc)	0.63	0.71	0.86	—	0.76	—	0.41	—	—	—	—
5-Methylcytosine	0.32	0.52	0.57	—	—	—	0.16	—	—	—	—

genose (XXIV) to give a mixture of anomers (Prystaš & Šorm, 1966b). However, in the case of 2,4-diethoxy-5-ethylpyrimidine (VI), which is a close analogue of 2,4--dimethoxy-5-methylpyrimidine, the course and yield of the reaction would be expected to be similar.

Reaction of VI with the protected chlororibose (XXIV) in anhydrous acetonitrile with molecular sieves was carried out in a sealed tube at 85°C, in the expectation that the increased temperature (and pressure) would accelerate the reaction rate and increase the yield. Reaction for 4 days under these conditions gave 1-(2,3,5--tri-*O*-benzoyl-β-D-ribofuranosyl)-4-ethoxy-5-ethyl-2(1H)-pyrimidinone (XXV) with a yield similar to that obtained at room temperature for 8 days, about 60%. Chromatography of the reaction mixture on Al_2O_3 (activity according to Brockman II - III) with 8:2 and 7:3 ratios (v/v) of benzene - ethyl acetate demonstrated the presence of only the β-anomer (XXV), which was isolated by thin-layer chromatography on Al_2O_3 and hydrolysed with HCl to give XXVI, the properties of which were identical with those described for 2′,3′,5′-tri-*O*-benzoyl-5-ethyluridine (Świerkowski & Shugar, 1969; Muraoka *et al.*, 1970). Treatment of this product with sodium methoxylate yielded the β-anomer of 5-ethyluridine (XXVII), m.p. 183 - - 184°C as compared to 184°C reported by Shapira (1962) and 185°C by Świerkowski & Shugar (1969), but in sharp contrast with the value of 158°C given by Muraoka *et al.* (1970). The same end product was also obtained by subjecting XXV to simultaneous debenzoylation and hydrolysis as follows: the acid eluate of the product of debenzoylation of the ester XXV on a Dowex 50W(H^+) column was simply left overnight, leading to hydrolysis of the 4-ethoxy substituent to give XXVII, the properties of which were identical with those obtained by the two-stage process.

Ammonolysis of the protected 4-ethoxy nucleoside (XXV) with anhydrous methanolic ammonia at 120°C yielded the required 5-ethylcytidine (β-anomer)

(XXVIIIa) which, on treatment with anhydrous HCl in ethanolic solution, precipitated as the crystalline HCl salt. Deamination of XXVIIIa with nitrous acid at room temperature gave 5-ethyluridine (XXVII), and hydrolysis of XXVIIIa with $HClO_4$ at 100°C yielded 5-ethylcytosine. The UV spectrum of XXVIIIa (Fig. 2b) was very similar to that of 5-methylcytidine (Fox & Shugar, 1952), pK_a for protonation of the ring N-3 slightly higher, and the chromatographic mobility considerably higher (Table 3).

It remains to add that phosphorylation of XXVIIIa to 5EtCDP has now been carried out and that this compound proved to be a substrate for polynucleotide phosphorylase. These findings, together with the properties of poly(5EtC) will be reported elsewhere (Kulikowski & Shugar, in preparation).

Treatment of XXV with methanolic methylamine and ethylamine was followed by the isolation of 5-ethyl-N^4-methylcytidine (XXVIIIb) and N^4,5-diethylcytidine (XXVIIIc), respectively. All three compounds, XXVIIIa,b,c exhibited spectral (Table 2), chromatographic (Table 3), and pK_a (Table 1) values similar to those presented above for 5-ethyl-1-methylcytosine and its N^4-alkyl derivatives (Tables 1, 2, 3).

Ultraviolet absorption spectra and dissociation constants

UV absorption spectra of 5-ethylcytosine and 5-ethylisocytosine are presented in Figs. 1a and 1b; of 5-ethyl-1-methylcytosine, 5-ethylcytidine, and their N^4-alkyl derivatives in Figs. 2a,b through 4a,b; of 1,N^4,N^4,5-tetramethylcytosine and N^4,N^4,5--trimethylcytosine in Figs. 5a and 5b; and 2,4-diethoxy-5-ethylpyrimidine and 5--ethyl-1-methyluracil in Figs. 6a and 6b. For simplicity, only the neutral and ionized forms are shown. For each compound the spectral titration to determine the pK_a value was based on the use of 7 buffers with pH values lying in the vicinity of the pK_a; the results, believed accurate to better than 0.05 pH units, are presented in Table 1. Previously published (Kulikowski *et al.*, 1969) spectral and pK_a data for 1,5-dimethylcytosine and its N^4-alkyl analogues were redetermined, using recrystallized compounds. Spectral and pK_a data for 1,5-dimethylcytosine were used as a control, our values for these being in excellent agreement with those reported by Fox *et al.* (1959). The spectrum of 5-ethylisocytosine (Fig. 1b), as expected, closely resembles that reported for isocytosine by Helene & Douzou (1964), due allowance being made for the bathochromic shift due to the 5-ethyl substituent; while the spectrum for 5-ethyl-1-methyluracil (Fig. 6b) is almost identical with that for 1-methylthymine (Wittenburg, 1966). The spectrum of 2,4-diethoxy-5-ethylpyrimidine (VI), obtained by Method B and shown in Fig. 6a, is also very similar to that for 2,4-diethoxy--5-methylpyrimidine (Wittenburg, 1966).

Note that the spectrum of 5-ethylcytosine (Fig. 1a) is practically identical with that for 5-methylcytosine (Shugar & Fox, 1952), as might have been anticipated; and that the spectra of the 1-methyl-5-ethylcytosine and its N^4-alkylamino derivatives (IXa,b,c) closely resemble those for the corresponding 1,5-dimethylcytosine and its N^4-alkylamino analogues (Kulikowski *et al.*, 1969) (Figs. 2a, 3a and 4a, Table 2).

The same applies to the corresponding ribonucleosides, with the exception of the N^4-alkyl-5-methylcytidines, which have hitherto not been described. However the previously described spectra (Kulikowski *et al.*, 1969) for the N^4-alkyl-5-methyl--2′-deoxycytidines provide an equally good comparison series.

Several regularities are immediately obvious on an examination of the N^4-alkyl derivatives of 5-methyl and 5-ethyl cytosines and cytidines (Table 2), as might be expected; e.g. the ratio $\varepsilon_{max}^{pH1}/\varepsilon_{max}^{pH7}$ decreases monotonously as one proceeds from

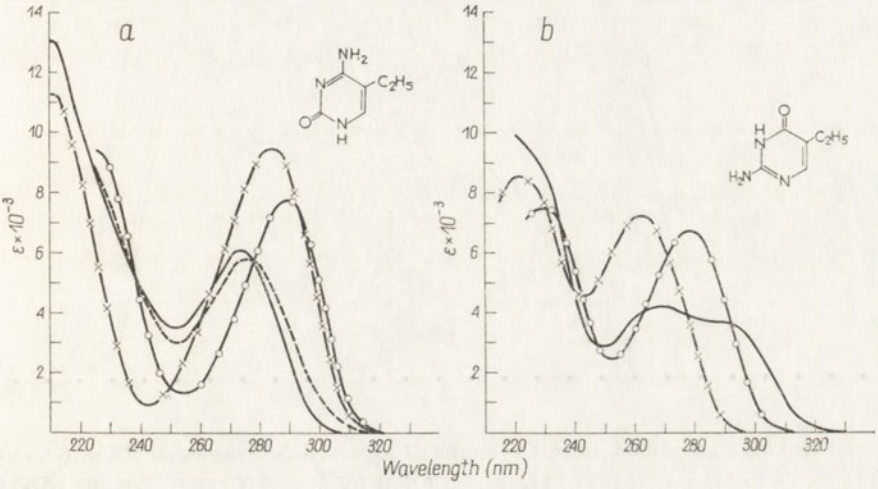


Fig. 1. Ultraviolet absorption spectra of: (*a*) 5-ethylcytosine (XIIa), (-×-×-×-), pH 1, cationic form; (——), pH 7, neutral form; (- - -), pH 12; (-○-○-○-), pH 14, anionic form; (*b*) 5-ethylisocytosine (XIIb), (-×-×-×-), pH 1, cationic form; (——), pH 7.2, neutral form; (-○-○-○-), pH 13, anionic form.

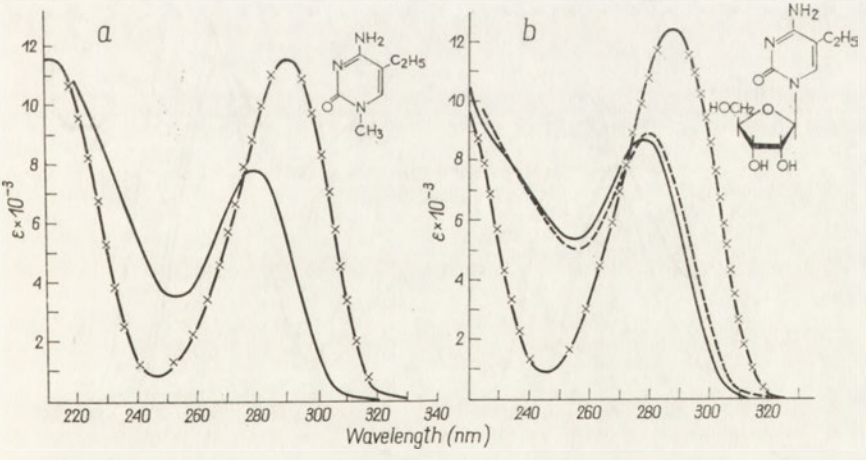


Fig. 2. Ultraviolet absorption spectra of: (*a*) 5-ethyl-1-methylcytosine (IXa), (-×-×-×-), pH 1, cationic form; (——), pH 7 - 14, neutral form; (*b*) 5-ethylcytidine (XXVIIIa), (-×-×-×-), pH 1, cationic form; (——), pH 7 - 12, neutral form; (- - -), pH 14, neutral form with dissociation of sugar hydroxyls.

$-NH_2$ to $-NHCH_3$ to $-NHC_2H_5$ to $-N(CH_3)_2$. Replacement of one of the amino hydrogens by an alkyl group does not result in any marked shift in λ_{max}. By contrast, introduction of a second alkyl leads to a pronounced bathochromic shift; while the spectral profile, particularly at short wavelengths, differs appreciably from those

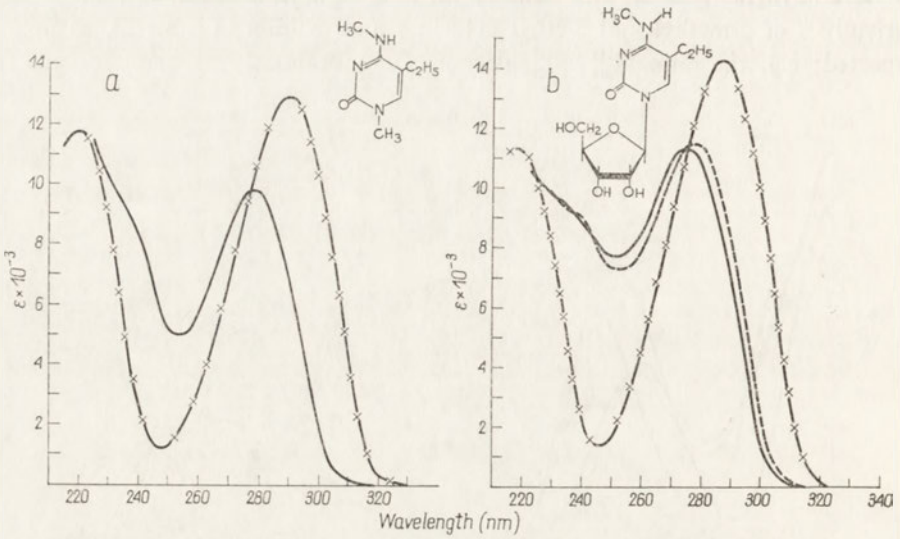


Fig. 3. Ultraviolet absorption spectra of: (*a*) 1,N^4-dimethyl-5-ethylcytosine (IXb), (-×-×-×-), pH 1, cationic form; (——), pH 7 - 14, neutral form; (*b*) 5-ethyl-N^4-methylcytidine (XXVIIIb), (-×-×-×-), pH 1, cationic form; (——), pH 7 - 12, neutral form; (- - -), pH 14, neutral form with dissociation of sugar hydroxyls.

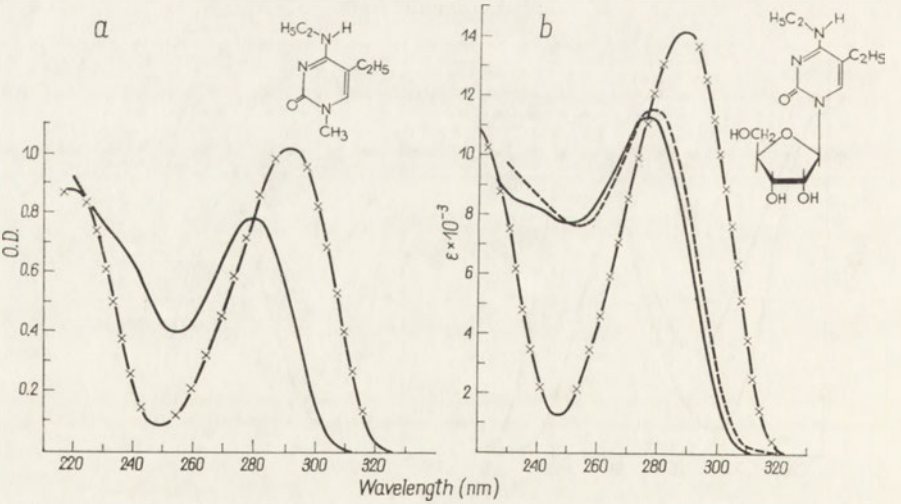


Fig. 4. Ultraviolet absorption spectra of: (*a*) N^4,5-diethyl-1-methylcytosine (IXc), (-×-×-×-), pH 1, cationic form;(——), pH 7 - 14, neutral form; (*b*) N^4,5-diethylcytidine (XXVIIIc), (-×-×-×-), pH 1, cationic form; (——), pH 7 - 12, neutral form; (- - -), pH 14, neutral form with dissociation of sugar hydroxyls.

for the 4-amino and 4-alkylamino derivatives. These findings are in accord with those of Martin & Reese (1967) and Shoup, Miles & Becker (1967), based on NMR spectra, according to which the N^4-alkyl derivatives of 1-methylcytosine and the corresponding ribo- and deoxyribosides exhibit hindered rotation of the amino group. The present results, based solely on electronic absorption spectra, demonstrate only that such hindered rotation of an exogenous alkylamino substituent is much more pronounced in the presence of a 5-alkyl substituent. However the electronic absorption spectra, which are a reflection of both inductive and steric effects, provide no concrete information as to the angle of rotation of an alkyl or dialkyl amino group with respect to the plane of the aromatic ring. It is planned to examine this problem with the aid of infrared and NMR spectroscopy.

5-Ethylcytosine and its 1,N^4-alkylamino derivatives, as well as 5-ethylcytidine

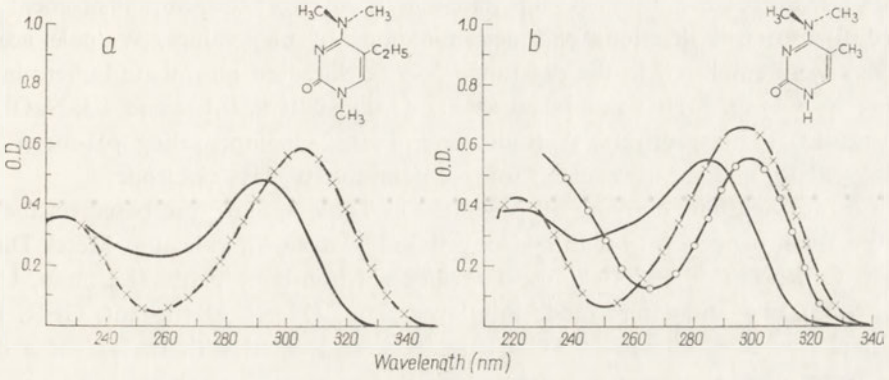


Fig. 5. Absorption spectra of: (*a*) 1,N^4,N^4,5-tetramethylcytosine (XXIIb), (-×-×-×-), pH 1, cationic form; (——), pH 7 - 14, neutral form; (*b*) N^4,N^4,5-trimethylcytosine (XXIII), (-×-×-×-), pH 1, cationic form; (——), pH 7, neutral form; (- - -), pH 14, anionic form.

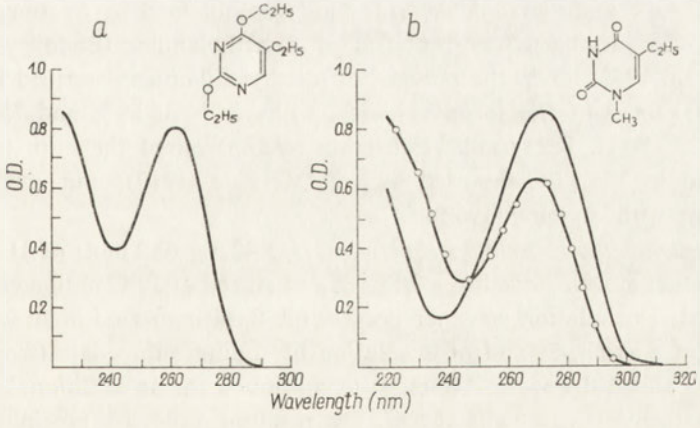


Fig. 6. Ultraviolet absorption spectra of: (*a*) 2,4-diethoxy-5-ethylpyrimidine (VI) at pH 7.2; (*b*) 5-ethyl-1-methyluracil (VIII), (——), pH 2 - 6, neutral form; (-○-○-○-), pH 12 - 14, anionic form.

and its N^4-alkylamino derivatives, exhibit slightly higher pK_a values (for protonation of the ring N-3 nitrogens) than the corresponding 5-methyl analogues. Since these differences are not large, they were measured directly for both series, confirming that the pK_a for the 5-ethyl series is 0.2 pH units higher than for the 5-methyl analogues (Table 1). A somewhat larger increase is shown by 5-ethylisocytosine, the pK_a for ring N-3 protonation of which is 4.7, hence 0.7 unit higher than for isocytosine; while the pK_a for dissociation of the N-1 hydrogen is 10.0, hence 0.6 unit higher than for isocytosine (Levene, Bass & Simms, 1926).

EXPERIMENTAL

Melting points were determined under a microscope hot stage (Beotius), with no correction. Ultraviolet absorption spectra were measured on a Zeiss (Jena) VSU 2-P instrument, using 10-mm path-length cuvettes; the same instrument was used for spectral titrations and determinations of pK_a values. Walpole acetate buffers were employed in the pH range 3 - 5.5; Sörensen phosphate buffers in the range 6 - 8.4; 0.1 N-HCl was taken as pH 1; and 0.01 N, 0.1 N and 1 N-NaOH as pH 12, 13, 14, respectively. A Radiometer PHM22 compensating pH-meter was employed for pH measurements, using a semi-micro glass electrode.

Chromatographic data are all presented in Table 3, and were based on the use of Whatman paper no. 1 (or thin-layer cellulose plates with indicator, Merck Darmstadt, GFR, TLC Plates Cellulose F), silica gel thin-layer plates (Eastman, USA, No. 6065), and aluminium oxide thin-layer plates (Merck Darmstadt, GFR, DC-fertigplatten Aluminiumoxid TF_{254}). Spots were located with the aid of a dark UV lamp.

5-Ethylbarbituric acid (I): This compound was prepared from barbituric acid by the procedure of Wenner (1946) in 90% yield, m.p. 175 - 180°C. Recrystallization from 50% ethanol gave a product with m.p. 193°C, as compared to 191°C reported by Wenner (1946), and λ_{max}^{pH12} 268 nm.

5-Ethyl-2,4,6-trichloropyrimidine (II): This was obtained by treatment of I with phosphorus oxychloride in the presence of dimethylaniline (Baddiley & Topham, 1944; Shapira, 1962), or in the presence of diethylaniline as described by Wempen & Fox (1963) for 2,4,6-trichloropyrimidine, with yields of 68% and 75% respectively, m.p. 72 - 74°C. Recrystallization from ethanol raised the m.p. to 74 - 76°C, as compared to 75 - 77°C reported by von Merkatz (1919); and $\lambda_{max}^{ethanol}$ 268 nm, in agreement with Shapira (1962).

6-Chloro-2,4-diethoxy-5-ethylpyrimidine (III): 42.3 g (0.2 mol) of II and 400 ml anhydrous ethanol were added to a 2 l flask and stirred at 30°C until a clear solution was obtained. The solution was then cooled, the flask immersed in an ice bath and, with constant stirring, 390 ml of a solution of sodium ethoxylate (from 15.8 g of sodium) was added dropwise. Mixing was continued for an additional 20 min and the solution then left overnight at 5°C. The resulting yellowish precipitate of NaCl was filtered off, the filtrate cooled to 2 - 3°C and, with constant stirring, 2.5 l water was added dropwise, followed by stirring for an additional 30 min at 2 - 5°C. Sub-

sequent storage overnight at 5°C led to the appearance of a heavy oil, which was separated out and dried over anhydrous sodium sulphate. Distillation of the oil gave a fraction of 27 g (60% yield) with a b.p. of 159°C (at 26 mm Hg), $\lambda_{max}^{ethanol}$ 267 nm, $\lambda_{min}^{ethanol}$ 240 nm. Analysis for $C_{10}H_{15}N_2O_2Cl$ gave (calculated): C, 52.07%; H, 6.56%; N, 12.15%; (found): C, 52.43%; H, 6.36%; N, 13.09%.

6-Chloro-5-ethyluracil (IV): To a round bottom 10-ml flask was added 461 mg (2 mmol) of III and 5 ml conc. HCl, and the mixture heated under reflux for 16 h. Hot water was then added to dissolve the precipitate, the solution left to cool to room temperature, and then stored overnight in the cold room. The resulting precipitate was filtered off, recrystallized from water and dried over P_2O_5 under vacuum at room temperature. Yield 245 mg (70%), m.p. 213 - 215°C, λ_{max}^{pH1} 267 nm, λ_{min}^{pH1} 235 nm; λ_{max}^{pH12} 290 nm, λ_{min}^{pH12} 250 nm. For $C_6H_7N_2O_2Cl$ (calculated): C, 41.3%; H, 4.0%; N, 16.4%; (found): C, 41.43%; H, 4.53%; N, 16.6%.

6-Amino-5-ethyluracil (V): To 175 mg (1 mmol) of IV in a 25 ml glass tube was added 15 ml ethanolic ammonia (saturated at 0°C), the tube sealed and heated for 20 h at 115 - 120°C. The contents of the tube were then brought to dryness under vacuum, and the residue twice recrystallized from water, with addition of activated carbon (Merck), and then dried under vacuum over P_2O_5 to give 112 mg (72%), m.p. 321 - 323°C, λ_{max}^{pH2} 274 nm, λ_{max}^{pH12} 276 nm.

Acid hydrolysis of V: A small sample of V in 1 N-HCl was heated under reflux for 1 h, and the solution then chromatographed on Whatman paper 3*MM* with solvent systems *A*, *B*, *C* (Table 3). A single spot was found, with R_F values and spectral properties corresponding to 5-ethylbarbituric acid (I).

2,4-Diethoxy-5-ethylpyrimidine (VI): (Method A): A vigorously stirred mixture of 23.1 g (0.1 mol) of IV, 90 g of zinc dust and 465 ml anhydrous ethanol was heated to 60°C, followed by dropwise addition of 178 ml conc. HCl over a period of 40 min, during which the temperature was maintained in the range 60 - 70°C. Stirring was continued at this temperature for a further 1.5 h, and the hot mixture filtered, followed by washing of the zinc with hot water. The combined yellow filtrates were concentrated under vacuum to a yellow oil, which was dissolved in a small volume of hot water, salted out with 500 ml of 33% KOH and steam-distilled to provide 650 ml mixture, to which was added 170 g K_2CO_3 and left overnight at room temperature. The oil which separated out was removed, and the remaining solution extracted with ether (450 ml). The ether extract was dried over sodium sulphate, the latter filtered off, and the extract distilled under reduced pressure. The fraction with a b.p. in the range 148 - 152°C (20 mm Hg) was collected to yield 13 g (76% yield), free of chlorine, and with λ_{max}^{pH7} 264 nm, λ_{min}^{pH7} 239 nm. As described above, this product contained 10% of known impurities, which, however, do not interfere with its use in subsequent Hilbert-Johnson reactions.

(Method B): A solution of 2,4-dichloro-5-ethylpyrimidine (XV), 1.77 g (0.1 mol) in 8 ml anhydrous ethanol, was slowly added, with stirring, to a solution of sodium ethoxylate (0.53 g in 9 ml ethanol). The mixture was heated under reflux for 10 min, cooled, and the sodium chloride filtered off and washed with ether. The combined filtrates were brought to dryness, the residue dissolved in 70 ml ether, and the ether

solution washed successively with 30% NaOH and water, dried over anhydrous sodium sulphate, and concentrated to an oil which was distilled at reduced pressure (110°C, 5 mm Hg) to give 1.67 g product (85% yield). The absorption spectrum is shown in Fig. 6a. For $C_{10}H_{16}N_2O_2$ (calculated): C, 61.22%; H, 8.16%; N, 14.3%; (experimental): C, 61.1%; H, 7.82%; N, 14.5%.

4-Ethoxy-5-ethyl-1-methyl-2(1H)-pyrimidinone (VII): To 0.59 g (3 mmol) of compound V was added 6.8 ml methyl iodide and the solution left at room temperature in the dark for two days, following which crystals of VII were present. The mixture was brought to dryness to give a light yellow residue, 502 mg (92%) after drying over P_2O_5, with m.p. 83 - 88°C. The crude product was recrystallized from ethyl acetate with addition of *n*-hexane to turbidity to yield 0.236 g, m.p. 88 - 91°C. Concentration of the filtrate, and further addition of *n*-hexane gave an additional 0.129 g, m.p. 84 - 91°C. An analytical sample was obtained by recrystallization from ether - ethanol, m.p. 91°C, $\lambda_{max}^{ethanol}$ 280.5 nm, $\lambda_{min}^{ethanol}$ 243 nm.

5-Ethyl-1-methyluracil (VIII): Recrystallized VII (0.23 g, 1.5 mmol) was dissolved in 4 ml conc. HCl and brought to dryness on a water bath. This operation was repeated and the residue dried under vacuum over KOH to provide 0.19 g (82% yield) of pale yellowish substance, m.p. 162 - 163°C. An analytical sample was obtained by crystallization from ethanol - ether, with m.p. 162°C, λ_{max}^{pH2-7} 273.5 nm, λ_{min}^{pH2-7} 238 nm; λ_{max}^{pH12} 271.5 nm, λ_{min}^{pH12} 245 nm.

5-Ethyl-1-methylcytosine (IXa): A mixture of 0.182 g (1 mmol) of VI and 15 ml ethanolic ammonia (saturated at 0°C) was heated in a 25 ml sealed tube at 120 - - 125°C for 40 h. The reaction mixture was brought to dryness and crystallized from methanol - cyclohexane to give 0.060 g, m.p. 245 - 246°C. The resulting filtrate was again brought to dryness and the residue crystallized again to give 0.074 g, m.p. 240 - 244°C, combined yield 0.134 g (80%). An analytical sample was obtained by recrystallization from methanol - benzene, m.p. 246°C, absorption spectrum as shown in Fig. 2a; λ_{max}^{pH1} 291 nm, ε_{max}^{pH1} 11.4×10^3; λ_{max}^{pH12} 280 nm, ε_{max}^{pH12} 7.72×10^3. The spectrophotometrically determined pK_a (for protonation of the ring N_3) was 4.95 ± 0.05.

1,N⁴-Dimethyl-5-ethylcytosine (IXb): Compound VI (0.18 g, 1 mmol) was subjected to amination in 15 ml of 40% solution of methylamine in anhydrous methanol at 120 - 125°C for 40 h. The solution was then brought to dryness and the residue crystallized from ethyl acetate, on addition of *n*-hexane to turbidity, to yield 0.12 g (65%). Recrystallization from ethyl acetate gave a product with m.p. 128 - 130°C, the UV spectrum of which is shown in Fig. 3a; λ_{max}^{pH1} 290 nm, ε_{max}^{pH1} 12.9×10^3, λ_{min}^{pH1} 247 nm; λ_{max}^{pH12} 278 nm, ε_{max}^{pH12} 9.75×10^3, λ_{min}^{pH12} 252 nm. The spectrally determined pK_a was 4.66 ± 0.05.

N⁴,5-Diethyl-1-methylcytosine (IXc): 0.182 g (1 mmol) of VI was treated with 15 ml of anhydrous 40% ethylamine in methanol as in the previous section, leading to 0.131 g (72%) of IXc, m.p. 99 - 101°C, λ_{max}^{pH1} 291 nm, ε_{max}^{pH1} 10.1×10^3; λ_{max}^{pH12} 278 nm, ε_{max}^{pH12} 7.80×10^3. The absorption spectrum is exhibited in Fig. 4a. Spectral titration led to a pK_a of 4.70 ± 0.05.

5-Ethyl-2-thiouracil (XIV): This was prepared as described by Burckhalter

& Scarborough (1955) in 30% yield, m.p. 190 - 192°C (the previous authors give 191 - 193°C), λ_{max}^{pH1} 277 nm, λ_{min}^{pH1} 241 nm.

5-Ethyluracil (X): (Method A): 20 ml (19.6 g, 0.1 mol) of 2,4-diethoxy-5-ethylpyrimidine (VI) was heated with 200 ml of conc. HCl under reflux for 16 h. Hot water was added to dissolve the precipitate and the solution left overnight in the cold room for crystallization. The crystals were removed and washed with water to remove traces of acid. Two recrystallizations from 70% ethanol gave 10.2 g (72% yield) needles, m.p. 286 - 288°C (decomp.). Further recrystallization from dilute ethanol solution provided an analytical sample, m.p. 298 - 300°C (decomp.), as compared to 300°C reported by Shapira (1962). λ_{max}^{pH1} 264.5 nm, λ_{max}^{pH12} 289 nm.

(Method B): Compound XIV was hydrolysed with chloroacetic acid as described by Buckhalter & Scarborough (1955). Recrystallization from 70% ethanol gave a product with m.p. 302 - 303°C in 76% yield, and spectral data as in Method A, above.

4-Ethoxy-5-ethyl-2(1H)-pyrimidinone (XIa): 9.81 g (0.05 mol) of VI was subjected to alkaline hydrolysis by the method of Hilbert & Jansen (1935) as modified by Szer & Shugar (1968). The sodium salt of XIa, which was deposited on cooling, was transformed to the free base and recrystallized from ethanol - ethyl acetate to give 1.8 g (20% yield), m.p. 158 - 160°C, λ_{max}^{pH7} 275 nm, λ_{min}^{pH7} 240 nm; λ_{max}^{pH13} 285 nm, λ_{min}^{pH13} 252 nm.

2-Ethoxy-5-ethyl-4(3H)-pyrimidinone (XIb): The mother liquors from the foregoing procedure were concentrated several times to yield, following transformation to the free base and recrystallization from ethanol - ethyl acetate, 2.1 g (24% yield). Recrystallization from ethanol gave m.p. 109 - 110°C, λ_{max}^{pH7} 262 nm, λ_{min}^{pH7} 238 nm; λ_{max}^{pH13} 268 nm, λ_{min}^{pH13} 245 nm. Further manipulation of the mother liquors from this preparation was accompanied by extensive decomposition, precluding the isolation of additional product.

2,4-Dichloro-5-ethylpyrimidine (XV): 28 g (0.2 mol) of 5-ethyluracil (X) and 120 ml phosphorus oxychloride were heated under reflux for 2 h, excess oxychloride removed under vacuum, and the syrupy residue poured, with vigorous stirring, into a mixture of ether - ice (40:260, v/v). The ether phase was removed and the aqueous phase extracted with 250, 100 and 100 ml portions of ether. The ether extracts were washed twice with saturated sodium carbonate and dried over anhydrous sodium sulphate. The ether was removed under vacuum and the resultant residue distilled under reduced pressure to yield 28 g (80%). Recrystallization from *n*-hexane gave a product with m.p. 51°C, $\lambda_{max}^{H_2O}$ 267 nm, $\lambda_{min}^{H_2O}$ 235 nm.

Amination of 2,4-dichloro-5-ethylpyrimidine (XV) to give 2-amino-4-chloro-5-ethylpyrimidine (XVIb) and 4-amino-2-chloro-5-ethylpyrimidine (XVIa): 1.77 g (0.01 mol) of XV and 16 ml of ethanolic ammonia (saturated at 0°C) were heated in a sealed tube for 5 h at 90°C. The precipitate, which separated out on cooling, was washed with cold ethanol and recrystallized from ethanol to yield 110 mg (7%) of 2-amino-4-chloro-5-ethylpyrimidine (XVIb), m.p. 178 - 179°C, λ_{max}^{pH1} 218 nm, 309 nm, λ_{min}^{pH1} 250 nm; λ_{max}^{pH7} 230 nm, 303 nm, λ_{min}^{pH7} 255 nm.

The filtrate from the foregoing operation was brought to dryness, the residue

dissolved in 5 ml conc. HCl, and the solution brought to dryness on a water bath. This operation was repeated and the dry residue recrystallized from ethanol to yield 1.14 g (72%) of 4-amino-2-chloro-5-ethylpyrimidine (XVIa), m.p. 146 - 147°C, λ_{max}^{pH1} 258 nm, λ_{min}^{pH1} 219 nm; λ_{max}^{pH7} 236 nm, 275 nm, λ_{min}^{pH7} 255 nm.

4-Amino-2-ethoxy-5-ethylpyrimidine (XVIIa): 1.9 g (0.012 mol) of XVIa was dissolved in 5 ml anhydrous ethanol, to which was added 5 ml of sodium ethoxylate in ethanol (from 0.29 g sodium). The mixture was heated for 5 h under reflux, cooled and filtered. The NaCl residue was washed with anhydrous ethanol and the combined filtrates brought to dryness to yield an oil which, on cooling, crystallized. Yield 1.14 g (88%). Recrystallization from *n*-heptane gave a product with m.p. 51 - 52°C, λ_{max}^{pH1} 225 nm, 265 nm, λ_{min}^{pH1} 242 nm; λ_{max}^{pH7} 224 nm, 276 nm, λ_{min}^{pH7} 246 nm.

2-Amino-4-ethoxy-5-ethylpyrimidine (XVIIb): The procedure for obtaining this compound was identical with that followed for XVIIa. On recrystallization from *n*-heptane, m.p. 57 - 58°C, λ_{max}^{pH1} 275 nm, λ_{min}^{pH1} 245 nm; λ_{max}^{pH7} 280 nm, λ_{min}^{pH7} 253 nm.

5-Ethylcytosine (XIIa): (Method A): 0.84 g (5 mmol) of XVIIa was dissolved in 12 ml conc. HCl and brought to dryness on a water bath. The residue was dissolved in water, ammonia added to precipitate the product in the cold and the precipitate filtered off. The product was once more dissolved in conc. HCl and brought to dryness under vacuum. The residue was recrystallized from ethanol to give the HCl salt of XIIa, 0.59 g (85%), m.p. 236.5°C (decomp.). For $C_6H_{10}ON_3Cl$ (calculated): C, 41.04%; H, 5.74%; N, 23.92%; found: C, 40.7%; H, 5.84%; N, 23.9%. λ_{max}^{pH1} 284 nm, ε_{max}^{pH1} 9.82×10^3, λ_{min}^{pH1} 242 nm; λ_{max}^{pH7} 274 nm, ε_{max}^{pH7} 6.08×10^3, λ_{min}^{pH7} 250 nm. Spectral titration gave a pK_a of 4.86 ± 0.05. The absorption spectrum is shown in Fig. 1a.

(Method B): 0.084 g (0.5 mmol) of XIa was aminated with 7 ml ethanolic ammonia (saturated at 0°C) in a sealed tube for 18 h at 125°C. The reaction mixture was brought to the boiling point, ethanol added to clarify the solution, insoluble particles filtered off, and the solution left overnight for crystallization. The following day 25 mg was filtered off. The filtrate was concentrated to 1 ml and, after standing overnight in the cold room, yielded an additional 23 mg. Yield, 70%, m.p. 270 - - 277°C. Recrystallization from water gave a product with m.p. 273 - 277°C (free base), as compared to 282 - 284°C reported by Johnson & Menge (1906). An analytical sample was dissolved in conc. HCl, brought to dryness under vacuum and recrystallized from ethanol to give the HCl salt, m.p. 236°C with chromatographic and spectral properties as for Method A, above.

5-Ethylisocytosine (XIIb): (Method A): 0.44 g (25 mmol) of XVIIb was dissolved in conc. HCl and brought to dryness on a water bath. The residue was dissolved in water, neutralized with NH_4OH and recrystallized from water to yield 0.31 g (70%), m.p. 230 - 232°C, λ_{max}^{pH1} 221 nm, λ_{min}^{pH1} 243 nm, ε_{max}^{pH1} 7.13×10^3. The absorption spectrum is shown in Fig. 1b.

(Method B): 0.162 g (1 mmol) of XIb was subjected to amination with 7 ml ethanolic ammonia, overnight at 125°C. The reaction mixture was then heated to boiling, filtered and stored in the cold room for crystallization, to yield 40 mg. Concentration of the mother liquor to 1 ml and cooling yielded an additional 50 mg

(total yield 65%), m.p. 228 - 230°C, unchanged on admixture of the product obtained by Method A, and with identical chromatographic and spectral properties.

2,4-Dichloro-5-methylpyrimidine (XIX): This was prepared by chlorination of thymine as elsewhere described (Schmidt-Nickels & Johnson, 1930; Bhat & Munson, 1968). Yield, 81%, m.p. 27 - 29°C, in agreement with Koppel *et al.* (1962).

2-Chloro-4-dimethylamino-5-methylpyrimidine (XX): Treatment of V with 33% aqueous dimethylamine according to Koppel *et al.* (1962) gave the title compound in 73% yield, m.p. 68 - 70°C, $\lambda_{max}^{ethanol}$ 258 nm, 289 nm.

4-Dimethylamino-2-ethoxy-5-methylpyrimidine (XXI): 0.85 g (5 mmol) of V was dissolved in 4 ml anhydrous ethanol, to which was added 4 ml sodium ethoxylate (from 0.24 g sodium) in ethanol. The mixture was heated under reflux for 6 h, cooled and filtered. The filtrate was brought to dryness under vacuum to yield 0.73 g (80%) of an oil, m.p. 12 - 14°C, which was redistilled under reduced pressure (5 mm Hg) to give again an oil which, on standing in the cold room, formed a white crystalline mass, m.p. 20.0-20.5°C.

1,N⁴,N⁴,5-Tetramethylcytosine (XXIII): 90 mg (1 mmol) of VI was added to 2 ml of methyl iodide and left at ambient temperature for 40 h. Excess methyl iodide was removed under vacuum at room temperature to leave an oil, to which was added 4 ml conc. HCl. Following evaporation on a water bath, the residue was dissolved in 5 ml water, filtered, the filtrate neutralized with NH_4OH and then brought to dryness under vacuum. The residue was crystallized from anhydrous ethanol to yield 41 mg with a pale yellow colour. Recrystallization from ethanol with addition of activated carbon gave a product with m.p. 194 - 197°C which was transformed to XXIIb with Ag_2SO_4 according to Hilbert (1934) to give an oil: λ_{max1}^{pH7} 225 nm, λ_{min}^{pH7} 255 nm, λ_{max2}^{pH7} 290 nm, $_{max1}^{pH1}$ 225 nm, λ_{min}^{pH1} 255 nm, λ_{max2}^{pH1} 305 nm; $\varepsilon_{max}^{pH1}/\varepsilon_{max}^{pH7}$ 1.241; $\varepsilon_{max2}^{pH1}/\varepsilon_{max1}^{pH1}$ 1.598; $\varepsilon_{max2}^{pH7}/\varepsilon_{max1}^{pH7}$ 1.316. The absorption spectrum is shown in Fig. 5a.

N⁴,N⁴,5-Trimethylcytosine (XXIIb): 90 mg (0.5 mmol) of VI in 5 ml conc. HCl was brought to dryness on a water bath. The residue was crystallized from anhydrous ethanol to yield 49 mg (60%) of the HCl salt. Recrystallization from ethanol gave an analytical sample, m.p. 156 - 159°C. λ_{max}^{pH1} 298 nm, ε_{max}^{pH1} 11.09×10^3, λ_{min}^{pH1} 252 nm; λ_{max}^{pH7} 285 nm, ε_{max}^{pH7} 9.78×10^3, λ_{min}^{pH7} 247 nm; λ_{max}^{pH14} 300 nm, ε_{max}^{pH14} 8.73×10^3, λ_{min}^{pH14} 265 nm. The absorption spectrum is shown in Fig. 5b.

1-O-Acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose (ABR): This was prepared as described by Ness, Diehl & Fletcher (1954), m.p. 130 - 131°C in agreement with the foregoing authors.

2,3,5-Tri-O-benzoyl-D-ribofuranose chloride (XXIV): Prepared as described by Yung & Fox (1963), and used immediately for subsequent reactions.

1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)-4-ethoxy-5-ethyl-2(1H)-pyrimidinone (XXV): The chloride (XXIV) prepared from 5.1 g of ABR was dissolved in 100 ml anhydrous acetonitrile in a 110 ml stainless steel autoclave, to which was added freshly prepared molecular sieves (BDH - 3A) and heated for 4 days at 82 - 85°C. The autoclave was cooled, the molecular sieves removed, and the reaction mixture

concentrated under vacuum to a syrup. Subsequent operations were performed by either of two methods:

(A): The syrup was dissolved in 50 ml benzene - ethyl acetate (7:2, v/v) and deposited on 40 plates (16×20 cm) covered with a 3-mm layer of neutral aluminium oxide (Merck neutral G or Bio-Rad AG7), activated for 1 h at 110 - 115°C. Development with benzene - ethyl acetate (7:2, v/v) gave three bands with R_F values 0.10, 0.50, 0.80. The band with R_F 0.50 was eluted with 50 ml ethyl acetate, the eluate brought to dryness under vacuum, and the resulting oil dissolved in ether and slowly evaporated under vacuum to yield a pseudocrystalline product which was crushed to a powder and dried over P_2O_5 under vacuum. Yield 3.52 g (58%), m.p. 60 - 63°C, $\lambda_{max}^{ethanol}$ 228 nm, 278 nm, $\lambda_{min}^{ethanol}$ 255 nm.

(B): The syrup was extracted with 40 ml petroleum ether and the residual oil dissolved in hot ethyl ether and filtered hot. Ether was removed under vacuum and the resulting oil dissolved in 40 ml hot ethyl acetate, to which was added petroleum ether to turbidity, and the mixture stored overnight in the cold room. The oil which separated out was removed, dissolved in hot cyclohexane, filtered and crystallization again attempted by storage overnight in the cold room. An oil was again obtained, and crystallization from cyclohexane once more attempted, but without success. The product was dried over P_2O_5 to give 2.27 g (37%) with a broad melting point. However, this product could be subjected to further operations such as amination.

2',3',5'-Tri-O-benzoyl-5-ethyluridine (XXVI): 375 mg (0.6 mmol) of XXV, acetic anhydride (300 mg) and anhydrous chloroform (30 ml) saturated with HCl at 0°C were mixed and stored for 16 h at room temperature. The reaction mixture was brought to dryness under vacuum and then distilled under vacuum from toluene and benzene. The residue was dissolved in boiling ethanol and hot hexane added to faint turbidity, following which the mixture was left for several hours at room temperature, then in the cold room to yield a precipitate of very small crystals in 87% yield, m.p. 153 - 155°C, as compared to 149.5 - 151.5°C reported by Świerkowski & Shugar (1969) and 154 - 155°C by Muraoka *et al.* (1970). $\lambda_{max}^{ethanol}$ 230 nm, 264 nm, $\lambda_{min}^{ethanol}$ 252 nm.

5-Ethyluridine (XXVII): (Method A): 235 mg (0.4 mmol) of XXV, 4 ml anhydrous methanol, and 2 ml 0.4 M-sodium methoxylate in methanol were added to a 25 ml flask. The mixture was left under anhydrous conditions for 12 h at room temperature, following which 1.6 g of Dowex 50W(H^+) was added, the mixture shaken for 1 h and the Dowex removed by filtration. The filtrate was brought to dryness under vacuum at 30°C, the residue dissolved in 20 ml anhydrous ethanol - - benzene and again brought to dryness under vacuum, to yield 110 mg of a light cream product. This was crystallized from ethanol by addition of hexane to turbidity to give 60 mg with a faint yellow tint, m.p. 185 - 188°C and a further 21 mg melting over a broad range (170 - 188°C), overall yield 75%. This product was further recrystallized by dissolution in hot ethanol in the presence of carbon (Merck, Carbo Medicinalis), filtration, addition to the filtrate of ethyl acetate to a faint turbidity, and storage in the cold room for several days. The resulting crystals ex-

hibited a m.p. of 183°C, compared to 184°C reported by Shapira (1962), 185°C by Świerkowski & Shugar (1969) and 158°C by Muraoka *et al.* (1970). Spectral properties were as reported by Świerkowski & Shugar (1969).

(Method B): 552 mg (0.9 mmol) of XXI, 5 ml anhydrous methanol and 7.5 ml of 0.4 M-CH_3ONa were well mixed and left for 14 h at room temperature, followed by addition of 3.9 g Dowex 50W(H^+), vigorous shaking for 30 min, and removal of the resin. The acid filtrate was left overnight at room temperature, brought to dryness under vacuum and evaporated with 25 ml water. The residue was extracted with 25 ml chloroform and washed with chloroform to give 172 mg (70%) of pale yellow solid with m.p. 177 - 180°C. This was dissolved in boiling ethanol, decolorized with carbon (BDH - acid washed) and hot hexane added to faint turbidity, yield 130 mg, m.p. 181 - 183°C.

5-Ethylcytidine (XXVIIIa): 1.96 g (3.2 mmol) of XXV, and 90 ml methanolic ammonia were heated in a stainless steel autoclave for 46 h at 120°C, and the methanol removed under vacuum. The brownish residue was dissolved in 100 ml water at 30 - 40°C with the aid of several ml ethanol and brought to dryness. The residue was dissolved in 150 ml water and extracted with 150, 100 and 100 ml portions of chloroform. During this operation a gummy substance collected at the interphase; this was filtered off. The aqueous phase was reduced to 100 ml under vacuum and extracted with 150, 100 and 100 ml portions of ether, then treated twice with active carbon and passed through a pad of Filter Cel. The filtrate was brought to dryness, dissolved in anhydrous ethanol and brought to dryness; and once again dissolved in ethanol, some benzene added, and brought to dryness. The resulting pseudocrystalline substance was dissolved in the minimal volume of hot anhydrous ethanol and poured, with stirring, into 200 ml acetone, to give a very fine precipitate. The suspension was left overnight at 5°C, filtered rapidly, and the precipitate washed with cold anhydrous acetone and dried over P_2O_5 to give crude 5-ethylcytidine (XXVIIIa) with a broad melting point, 100 - 108°C.

The crude product was deposited on a 2×5 cm column of Dowex 1×2(OH^-), and eluted with water, fractions of 15 ml being collected at intervals of 8 min. Fractions 10 - 36, exhibiting intense absorption at 278 nm, were combined and brought to dryness under vacuum, and the residue distilled twice from anhydrous ethanol-benzene. The residue was dissolved in anhydrous ethanol, cooled to 0°C and saturated with dry HCl to precipitate 210 mg of white crystals, m.p. 174 - 176°C. Recycling of the mother liquor in the same way gave an additional 130 mg crystals, m.p. 169 - 172°C.

The mother liquor remaining after precipitation of crude XXVIIIa, above, was brought to dryness, dissolved in hot anhydrous ethanol, slightly cooled, and saturated with HCl. Some precipitate formed during this operation. The mixture was then stored overnight in the cold room, the precipitate removed, washed with cold anhydrous ethanol and dried under vacuum, first over KOH, then P_2O_5, to yield 171 mg of colourless crystals of 5-ethylcytidine HCl, m.p. 174.2 - 175.8°C. The mother liquor from this operation was reduced to one-half volume, saturated with HCl and stored overnight in the cold to yield an additional 73 mg, m.p. 170 -

- 172°C. The mother liquor from this latter operation was reduced to one-half volume and left overnight in the cold to yield 33 mg crystals, m.p. 167.5 - 170.5°C. Overall yield 68% with respect to XXV. Recrystallization of the HCl salt from anhydrous ethanol gave a product with m.p. 176 - 177.5°C (decomp.), λ_{max}^{pHl} 288.5 nm, ε_{max}^{pHl} 12.4×10^3, λ_{min}^{pHl} 245.5 nm; $\lambda_{max}^{pH7\text{-}12}$ 277.9 nm, $\varepsilon_{max}^{pH7\text{-}12}$ 8.75×10^3, $\lambda_{min}^{pH7\text{-}12}$ 256 nm. Spectral titration gave a pK_a of 4.42 ± 0.05. The absorption spectra are shown in Fig. 2b. For $C_{11}H_{18}N_3O_5Cl$ (calculated): C, 42.9%; H, 5.87%; N, 13.65%; found: C, 43.2%; H, 5.92%; N, 13.7%.

Deamination and hydrolysis of XXVIIIa: 20 mg of XXVIIIa was dissolved in 4 ml of 2 N-acetic acid, 80 mg $NaNO_2$ added and the mixture kept at room temperature for 40 h. It was then brought to dryness and the residue extracted with ethanol. The extract was deposited as a band on Whatman 3*MM* and run with *sec.*-butanol - water (upper phase). The only spot, corresponding to 5-ethyluridine (XXVII) was eluted with water, the eluate brought to dryness, and the residue crystallized from ethanol - ethyl acetate to give crystals with m.p. 183°C, unchanged on admixture of XXVII.

Following hydrolysis of a 5 mg sample of XXVIIIa with 72% perchloric acid at 100°C for 1 h, the sole product was 5-ethylcytosine (XIIa), as shown by chromatography and spectral properties.

5-Ethyl-N⁴-methylcytidine (XXVIIIb): 0.153 g (0.25 mmol) of XXV was treated in a sealed tube with 15 ml of 40% methylamine in anhydrous methanol for 48 h at 125°C. The reaction mixture was brought to dryness, and the residue dissolved in water and rapidly brought to dryness. This operation was repeated and the residue dissolved in 25 ml water and extracted with 2 portions of 25 ml chloroform. The aqueous, yellowish, phase was treated with active carbon and the resulting almost colourless solution concentrated to small volume, deposited on a 1×5 cm column of Dowex $1\times2(OH^-)$, and eluted with water. The fractions absorbing at 278 nm were combined, brought to dryness, the residue dissolved in anhydrous ethanol and saturated with HCl at 0°C. The precipitate which formed was removed, washed with cold anhydrous ethanol and dried over KOH to give 38 mg of crystals, m.p. 182 - 184°C. The mother liquor was concentrated to one-half volume, again saturated with HCl and left overnight in the cold room to yield 23 mg, m.p. 178 - 182°C. Total yield 72%. An additional recrystallization of the HCl salt of XXVIIIb from anhydrous ethanol - ethyl acetate gave a product with m.p. 183 - 184°C, the spectrum of which is shown in Fig. 3b; λ_{max}^{pHl} 287 nm, ε_{max}^{pHl} 14.3×10^3, λ_{min}^{pHl} 246 nm; λ_{max}^{pH12} 275 nm, ε_{max}^{pH12} 11.2×10^3, λ_{min}^{pH12} 251.5 nm. Spectrally determined pK_a 4.14 ± 0.05.

N⁴,5-Diethylcytidine (XXVIIIc): 306 mg (0.5 mmol) XXI was heated in sealed tube with 20 ml of 40% anhydrous methanolic ethylamine at 120°C for 64 h. The mixture was brought to dryness, dissolved in 50 ml water and again brought to dryness. The operation was repeated, the residue dissolved in 25 ml water and extracted 4 times with 25 ml portions of chloroform. The yellowish aqueous phase was treated with carbon, brought to dryness, and the residue dissolved in anhydrous ethanol, which was then saturated with HCl at 0°C. The solution was brought to dryness and the residue crystallized from anhydrous ethanol to give, following

washing of the crystals with cold ethanol and drying over KOH, 41 mg, m.p. 176°C. The concentrated mother liquor was again saturated with HCl and left overnight in the cold room to yield 57 mg, m.p. 176°C (total yield 65%). Recrystallization from ethanol, and drying of the crystals over P_2O_5, at 100°C, gave a product with m.p. 182 - 183°C, and spectrum exhibited in Fig. 4b, with λ_{max}^{pHI} 288 nm, ε_{max}^{pHI} 13.8 × $\times 10^3$, λ_{min}^{pHI} 247 nm; λ_{max}^{pH12} 276 nm, ε_{max}^{pH12} 11.0 × 10^3, λ_{min}^{pH12} 252 nm. Spectral titration gave a pK_a value of 4.16±0.05.

This project, and related studies, have been supported in part by the World Health Organization, The Wellcome Trust, and the Agricultural Research Service, U.S. Dept. of Agriculture

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ANALOGI CYTOZYNY I CYTYDYNY: SYNTEZA I WŁASNOŚCI 5-ETYLOCYTOZYNY, 5-ETYLOCYTYDYNY ORAZ SZEREGU ICH POCHODNYCH

Streszczenie

1. Opisano syntezę i własności 2,4-dwuetoksy-5-etylopirymidyny oraz szeregu jej pochodnych.

2. Związki te są substratami do syntezy niektórych związków modelowych, takich jak 5-etylo--1-metylocytozyna oraz jej N^4-alkilopochodne. Opisano własności tych związków.

3. Otrzymano również związki modelowe z serii 5-metylocytozyny: N^4,N^4,5-trójmetylocytozynę i 1,N^4,N^4,5-czterometylocytozynę.

4. Opracowano nową wydajną metodę syntezy 5-etylocytozyny. W syntezie tej otrzymuje się jako produkt uboczny 5-etyloizocytozynę.

5. Stwierdzono, że wprowadzenie podstawnika 5-etylowego do 2,4-dwuchloropirymidyny w znacznym stopniu uprzywilejowuje pozycję 4 tego związku w przypadku nukleofilowego ataku amoniaku.

6. Opracowano metodę syntezy 5-etylocytydyny i jej niektórych N^4-alkilopochodnych opartą na przegrupowaniu Hilberta-Johnsona. Metoda ta umożliwia jednoczesne otrzymywanie z dobrą wydajnością 5-etylourydyny.

7. Przedstawiono i poddano dyskusji widma oraz wartości pK_a wyżej wymienionych związków.

8. Omówiono potencjalne biologiczne znaczenie analogów: 5-etylocytozyny i 5-etylocytydyny.

Received 12 January, 1971.

ACTA BIOCHIMICA POLONICA
Vol. 18 1971 No. 2

RECENZJE KSIĄŻEK

H. Sajonski und A. Smollich, ZELLE UND GEWEBE. S. Hirzel Verlag, Leipzig 1969; str. 256, cena DM 33.90.

Omawiana książka ma podtytuł: „Wprowadzenie dla medyków i przyrodników". Jest też zgodnie z tym podtytułem krótkim i przejrzystym podręcznikiem cytologii ogólnej i histologii. Zagadnienia w niej poruszane oparto na współczesnym ujęciu nauki o komórkach żywych i tkankach, łącząc w sposób zwięzły ale przystępny omawianie struktury morfologicznej z biochemicznymi podstawami funkcjonowania opisywanych komórek i tkanek.

Książka składa się z trzech większych części: pierwsza przedstawia krótki zarys histologicznych metod badawczych, druga dotyczy komórki, trzecia tkanek. Po każdej z tych części jest podany wykaz ważniejszej literatury monograficznej, przeglądowej, a niekiedy także pozycji piśmiennictwa archiwalnego; wybór piśmiennictwa obejmuje głównie nowe pozycje aż do roku 1968 i jest dokonany z myślą o czytelniku, który zechce rozszerzyć sobie podstawowe wiadomości zawarte w omawianym „wprowadzeniu". Autorzy podają nowoczesny pogląd na metody mikroskopowego badania tkanek, wspominając m. in. nie tylko o możliwościach, jakie daje mikroskopia elektronowa, ale także i elektronowa mikroskopia skaningowa. Część książki dotycząca komórki zawiera nie tylko omówienie budowy ważniejszych tworów wewnątrzkomórkowych ale także ich funkcji; przedstawiono również ważniejsze ciągi metaboliczne, proces biosyntezy białka oraz niektóre inne zagadnienia molekularnych podstaw cytologii. Przegląd tkanek zawarty w trzeciej części omawianego podręcznika jest bogato ilustrowany fotografiami z mikroskopu świetlnego, z mikroskopu elektronowego, oraz licznymi schematami, co jest szczególnie cenne dla czytelnika wchodzącego od podstaw w tajniki budowy żywych komórek i tkanek. Szkoda, że wszystkie ilustracje wykonane są w technice czarno-białej. Barwne fotografie i schematy uczyniłyby podręcznik Sajonskiego i Smollicha jeszcze atrakcyjniejszym.

Oceniający jest zdania, że w omawianym podręczniku zachowano właściwą proporcję pomiędzy dążnością do wprowadzania nowych szczegółów i faktów a rozmiarami książki przeznaczonej dla studentów do opanowania materiału w stosunkowo krótkim czasie. Jakkolwiek więc chciałoby się widzieć w niej nieco więcej wiadomości o współczesnej cytologii (np. autorzy nic nie mówią o preparatywnym otrzymywaniu i badaniu izolowanych organelli wewnątrzkomórkowych, nie wspominają także o formacji GERL — Golgi Ergastoplasmic Reticulum Lysosomes), to jednak w całości książka stanowi bardzo dobry podręcznik, łączący w umiejętny sposób wiadomości o budowie komórek i tkanek z podstawowymi wiadomościami o ich funkcji i biochemicznej aktywności. Z pewnością jest to cenna pozycja podręcznikowa.

Mariusz Żydowo

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS. Vol. XI, part 1 (R.T. Holman, ed.). R.M. Williams & D. Chapman: *Phospholipids, Liquid Crystals and Cell Membranes*. V. Mahadevan: *Chemistry and Metabolism of Fatty Aldehydes*. C. R. Smith: *Occurrence of Unusual Fatty Acids in Plants*. Pergamon Press, Oxford, New York, Toronto Sydney, Braunschweig 1970; str. 177, cena 55/-, $ 7.40.

Kolejny tom wydawnictwa dedykowany jest pamięci zmarłego w 1969 r. Johna B. Browna, wybitnego badacza lipidów, głównie kwasów tłuszczowych. Krótki życiorys prof. Browna zamieszczony jest na początku książki.

Pierwsza z trzech zawartych w tomie monografii dotyczy badań nad fosfolipidami w środowisku bezwodnym oraz w układzie fosfolipidy - woda (analiza termiczna, spektroskopia w podczerwieni, analiza rentgenowska i inne). Badania dotyczą lipidów zarówno syntetycznych, jak i uzyskanych ze źródeł naturalnych. Przedstawiono poglądy na ułożenie cząsteczek fosfolipidów w stanach mezomorficznych (przejściowych między stanem krystalicznym a ciekłym, czyli tzw. ciekłych kryształów) oraz omówiono mezomorfizm liotropowy fosfolipidów (stan przejściowy przed rozpuszczeniem w wodzie). Dużo miejsca poświęcili autorzy zagadnieniu wpływu budowy chemicznej fosfolipidów oraz obecności innych związków, jak cholesterol lub białka, na powstawanie różnych faz liotropowych: heksagonalnej, warstw jednocząsteczkowych i innych. Przy omawianiu układów fosfolipidy - woda autorzy nawiązują do zagadnienia błon biologicznych. W osobnym rozdziale omówione są dość krótko funkcje biologiczne fosfolipidów i sprecyzowane ich znaczenie w najważniejszych procesach życiowych. Szczegółowe dane wraz z obszernym piśmiennictwem (razem 226 pozycji, w tym wiele prac własnych) czynią z omówionego artykułu podstawową pracę dla wszystkich pragnących zapoznać się z fizykochemią fosfolipidów.

Następna monografia przedstawia współczesny stan wiedzy o długołańcuchowych aldehydach alifatycznych. Autorzy omawiają różne rodzaje syntezy chemicznej, reakcje charakterystyczne oraz metody oznaczania tych wybitnie czynnych chemicznie związków. Aldehydy łańcuchowe występują w przyrodzie głównie jako składniki plazmalogenów. W pracy zreferowane są metody izolowania wolnych i związanych aldehydów ze źródeł naturalnych. Bardzo ciekawy dla biochemika, chociaż znacznie krócej potraktowany niż część chemiczna, jest rozdział o metabolizmie aldehydów długołańcuchowych. Piśmiennictwo obejmuje 316 pozycji, z których większość dotyczy części chemicznej.

Ostatnia monografia omawia rzadziej spotykane kwasy tłuszczowe (inne niż nasycone, olejowy, palmitoolejowy, linolowy i linolenowy). Bardzo duża ilość tych kwasów odkryta została w ciągu ostatnich dziesięciu lat, najwięcej w nasionach roślin oleistych. Praca zawiera przejrzyste tablice obejmujące wszystkie omawiane kwasy z symbolicznie podaną budową lub wzorem, ich źródła naturalne oraz piśmiennictwo. Monografia nie obejmuje biosyntezy omówionych kwasów, natomiast autor zapowiada oddzielny artykuł na ten temat w tym samym wydawnictwie. Piśmiennictwo obejmuje 263 pozycje.

Hanna Wehr