POLSKA AKADEMIA NAUK
KOMITET BIOCHEMICZNY I BIOFIZYCZNY

POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. XI
No. 1

WARSZAWA 1964
PAŃSTWOWE WYDAWNICTWO NAUKOWE

ADDRESS

Krakowskie Przedmieście 26/28, Warszawa 64,
Poland

Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakł. 1906+154 egz. Ark. wyd. 6,25 ark. druk. 5,375+0,375+1 wkl.
Papier druk. sat. kl. III, 80 g. 70×100
Oddano do składania 27.X.1963. Podpisano do druku 2.II.1964.
Druk ukończono w lutym 1964.
Zam. 435/63 Z-20 Cena zł 25.—

Warszawska Drukarnia Naukowa — Warszawa, Śniadeckich 8

UNIVERSITATI CRACOVIENSI

QUAE PER SEX SAECULA CONDITORIS SUI CASIMIRI MAGNI POLONIAE REGIS MANDATO UT FONS DOCTRINARUM IRRIGUUS DE CUIUS PLENITUDINE HAURIRENT UNIVERSI LIBERALIBUS CUPIENTES IMBUI DOCUMENTIS FIERET FIDELIS EXSTITIT

PIO ANIMO OFFERUNT

Auctores Editores et Redactores
Actorum Biochimicorum Polonicorum

ACTA BIOCHIMICA POLONICA

Vol. XI 1964 No. 1

M. KUJAWSKI

IMPROVED METHOD FOR PREPARATION OF LIMIT DEXTRIN SUBSTRATE BY USING BARLEY MALT

Department of Agricultural Technology, College of Agriculture, and Department of Plant Physiology, Polish Academy of Sciences, Kraków

Limit dextrins are a mixture of branched oligosaccharides remaining after the α- and β-amylolytic hydrolysis of amylopectin, and they are not fermented by yeast. Besides the α-1,4-glycosidic links, they contain a considerable number of α-1,6-links, and they are the specific substrate for the estimation of limit dextrinase (dextrin-6-glucanohydrolase, E.C. 3.2.1.10), as this enzyme does not decompose bacterial dextran and is only slightly active toward isomaltose. The limit dextrinase is estimated by incubating the enzyme extract with limit dextrin substrate and measuring the reducing sugars formed. However, the specificity of this method is not very high, as the action of other α-1,6-glucosidases or transglucosidases cannot be excluded. The possibility of the α-1,4-links being attacked by amyloglucosidase should also be considered, and if rather long chains are present, secondary splitting even by malt β-amylase may occur. The term dextrinolytic ability used by Klimowski & Rodzewicz [1] seems therefore to be more correct, similarly as the term dextrinolytic activity used in this paper.

For an accurate estimation of limit dextrinase, the preparation of an appropriate limit dextrin substrate is therefore essential. This substrate should be rich in α-1,6-links, have a low degree of polymerization, and be consequently less susceptible to the action of α-1,4-glucosidases.

The methods for the preparation of limit dextrins were reviewed by Redfern [5]; all of them are based on four main steps: hydrolysis of starch by purified preparations of α- and β-amylases, or by barley malt; fermentation by yeast to remove the fermenting sugars; concentration and purification of the fluid remaining after fermentation; and precipitation of the dextrins with alcohol.

The method of Pronin [4] for preparation of limit dextrins based on the application of barley malt did not give satisfactory results. The 4-day-old malt decomposed starch paste very slowly, and the disap-

pearance of positive iodine reaction was often observed not earlier than after 12 hr. The obtained preparation of dextrins gave always a violet-brown colour with iodine indicating the presence of longer chains which make these dextrins an unsuitable substrate for the estimation of dextrinolytic activity. Redfern [5] also pointed out that "the use of barley malt for preparation of the limit dextrin probably does not give as well defined a substrate as the Kneen method..."

The present paper describes an improved method for preparation of limit dextrins based on the use of brewer's malt. The utility of the preparations as specific substrate for limit dextrinase from barley malt and from *Aspergillus oryzae* was also tested.

EXPERIMENTAL

Preparation of limit dextrins

The modifications introduced to the method of Pronin [4] were checked in preliminary experiments; for the sake of clarity, only the final procedure is reported.

Materials. Commercial potato starch "Superior" was used; 6% starch paste was prepared by mixing 2500 ml. of distilled water with 150 g. of starch, autoclaving the mixture for 1 hr. at 1.75 atm. overpressure and then cooling it to 37°.

For the hydrolysis of starch, barley malt extract was used. Seven-day-old brewer's malt, 40 g., was ground and mixed with 200 ml. of water, then extracted for 1 hr. at 40° with continuous stirring, cooled to room temperature and filtered.

Saccharification. To the starch solution, 140 ml. of malt extract was added and incubated at 37° for 2-2.5 hr. After this time no colour with iodine was observed. The solution was then cooled and filtered to remove impurities and the residue of the non-decomposed fraction.

Fermentation. Per approx. 2800 ml. of the solution, 50 g. of compressed yeast was taken, and the fermentation performed at 28-29° during 3 days. After this time no more formation of CO_2 was observed.

Isolation. The fluid after fermentation was adjusted to pH 6.5-7.0 and quickly brought to boil to precipitate proteins, then cooled and filtered. A slightly opalescent solution was concentrated on a boiling water bath to a volume of about 100 ml. The brown-red syrup thus obtained was filtered again giving a thick but clear fluid. For the precipitation of dextrins, to 100 ml. of the syrup 150 ml. of 96% ethanol (1.5 × vol. of the syrup) was poured slowly from a separatory funnel, with vigorous stirring. Under these conditions the dextrins of higher degree of polymerization were precipitated to form a sticky brown tar.

After 12 hr. the clear supernatant was carefully poured off. The lower limit dextrins present in the solution were precipitated by 80% ethanol concn., the solution of dextrins being poured into 350 ml. (3.5 vol. of the original vol. of the syrup) of 96% ethanol, with continuous stirring. The precipitated light-brown dextrins had a thick, gummy consistency. After 24 hr., the alcohol was poured off, and the sediment transferred into a mortar containing 96% ethanol. It was fragmented there into small pieces and after 12 hr. ground until a fine powder was obtained. The dextrins were separated on a Büchner funnel, washed with acetone and dried at room temperature in a thin layer.

Analysis of the obtained limit dextrins

Seven separate preparations of limit dextrins were analysed. All the estimations were performed on two parallel samples. The results were calculated per 100 g. of dry weight of the preparation (Table 1).

Dry weight. The content of water was estimated by drying approx. 1 g. of dextrins for 3 hr. at 105°.

Colour of 1% solution. The solution used as standard consisted of 0.6 ml. of 0.1 N-$K_2Cr_2O_7$ made up to 100 ml. with distilled water. To 100 ml. of the standard, 0.1 N-solution of iodine was added from a microburette and the colour compared with that of the dextrin solution. The results were expressed in milliliters of the 0.1 N-iodine solution added.

Phosphorus. The method of Fiske & Subbarow in the modification of Müller [2] was used. Inorganic P was assayed directly, and total P after digestion of the sample with conc. sulphuric acid. From these values, organic P was calculated.

Reducing power. Three methods were used: the iodometric method of Wilstätter described by Pawlowski - Doemens [3], the copper method of Bertrand and the ferricyanide method of Strepkow [8]. For the estimations, 1% solution of dextrins was prepared. The results expressed as glucose were calculated per 100 g. of dry weight of dextrins.

Purity of the preparation. This was estimated from the amount of glucose present after hydrolysis. Hydrolysis of dextrins was performed according to Pronin [4] using 10 ml. of the 1% solution, 40 ml. of water and 6 ml. of 20% HCl. After neutralization, glucose was estimated by the three above mentioned methods.

Degree of polymerization. This was calculated basing on the estimation of the reducing power of dextrins and on the content of glucose after hydrolysis.

Yield. This was calculated from the quantity of starch used and that of the obtained dextrins.

Table 1

Some chemical properties of the obtained preparations of limit dextrins

Colour of 1% solution is expressed in ml. of 0.1 N-iodine solution. Other results are calculated per 100 g. dry weight.

No. of preparation	Yield (%)	Dry wt. (%)	Colour (ml. of 0.1 N-iodine)	Phosphorus (%)			Reduction (glucose %)			Glucose after hydrolysis (%)			Degree of polymerization		
							Method of estimation								
				total	inorganic	organic	ferricyanide	iodometric	copper	ferricyanide	iodometric	copper	ferricyanide	iodometric	copper
1	2.8	92.36	0.12	0.260	0.099	0.161	17.85	12.26	9.10	93.40	89.88	76.32	5.2	7.3	8.3
2	4.3	92.86	0.10	0.225	0.074	0.151	18.57	12.96	9.43	91.08	93.35	83.00	4.9	7.2	8.8
3	3.6	92.76	0.18	0.213	0.054	0.159	19.52	13.64	10.35	85.89	93.05	82.90	4.4	6.8	8.0
4	3.9	92.21	0.14	0.209	0.073	0.136	16.73	12.42	8.63	85.41	92.28	76.26	5.1	7.4	8.8
5	3.5	92.56	0.14	0.237	0.080	0.157	16.77	12.20	8.76	87.73	91.49	76.36	5.2	7.5	8.7
6	2.8	92.76	0.30	0.194	0.034	0.160	17.17	12.88	10.05	83.99	92.17	76.61	4.9	7.2	7.6
7	2.7	91.92	0.22	0.269	0.115	0.154	17.27	12.28	9.40	84.72	93.00	78.15	4.9	7.6	8.3

Estimation of the dextrinolytic activity

To check the reproducibility of biochemical properties in successive preparations of limit dextrins, the dextrinolytic activity of enzyme extracts from malt and from mould was estimated. The assays were done by the method described by Pronin [4] taking into account the data of Schwarz & Malsch [7].

Enzyme extracts were prepared in the following way. Seven-day-old brewer's malt, 10 g., was extracted for 1 hr. with 250 ml. of water at 40°, with continuous stirring. Then the extract was filtered. For the experiments, two separate preparations were used. *Aspergillus oryzae* was cultivated on wheat bran; 10 g. of the dried mould bran was extracted at 40° with 250 ml. of 0.1% NaCl solution for 1 hr., with stirring, and filtrated. Three separate extracts were used for the experiments.

To prepare the substrate, 1 g. of dextrins was dissolved in 80 ml. of water, 10 ml. of 1/15 M-KH_2PO_4 was added, and the solution made up to 100 ml. with water, the pH being then 6.6-6.9. In some experiments the solution of dextrins was adjusted with citric acid to pH 5.0 as checked by a pH-meter.

Next, 10 ml. of 1% solution of limit dextrins was incubated with 5 ml. of malt extract (or 2.5 ml. of mould bran extract) for 2 hr. at 55°.

Table 2

Estimation of dextrinolytic activity with different preparations of limit dextrins

1% solution of dextrins was used; when it was buffered with KH_2PO_4, the final pH is given in the Table; in one series of experiments pH was adjusted to 5.0 with citric acid. The dextrinolytic activity is expressed in milligrams maltose formed from the limit dextrins by the action of 1 g. malt (or 1 g. mould bran) at 55° during 1 hr.

Dextrin prepar. no.	pH	Dextrinolytic activity						
		Malt extracts		*Aspergillus oryzae* extracts				
		I	II	I	II	III	III	
1	6.75	1.7	0.0	113	133	128	pH 5.0	–
2	6.60	3.4	3.4	137	156	147		178 178
3	6.60	0.0	3.4	133	152	144		–
4	6.75	1.7	0.8	127	130	132		171 173
5	6.85	0.0	0.0	120	123	127		167 167
6	6.80	1.7	1.7	125	130	140		–
7	6.85	1.7	1.7	118	120	135		–

The reaction was stopped by adding 0.2 ml. of 1 N-NaOH (the thymolphthalein indicator should then be blue). The control was prepared in the same way except that the solution of NaOH was added (after 2 hr.) before the addition of the enzyme extract. The amount of sugar was estimated iodometrically [3], 0.04 N-sodium thiosulfate being used for the titration. The differences between two parallel assays did not exceed 0.1 ml. of $Na_2S_2O_3$. The dextrinolytic activity was expressed in milligrams maltose formed from the limit dextrins by the action of 1 g. malt (or 1 g. mould bran) at 55° during 1 hr. (Table 2).

DISCUSSION

The described procedure for obtaining limit dextrins introduces several modifications to the method of Pronin [4]. Rather extensive hydrolysis of starch and its rapid progress were thus assured, and the obtained limit dextrins exhibited a high degree of purity and suitable biochemical properties.

The solubilization of the starch in steam at 1.75 atm. and the use of 7-day-old brewer's malt permitted to reduce the time of saccharification to 2 hr. The use of 7-day-old brewer's malt might seem disputable as it is known that only 1 to 4-day-old malt contains practically no limit dextrinase. However, a rapid inactivation of the enzyme takes place when brewer's malt is being dried at temperatures exceeding 70°, and in dry malt only very small quantities of the enzyme were found [5]. The dextrinolytic activity of the dried brewer's malt used in the present study was also practically nil. Moreover, at the temperature used for saccharifying the starch solution (35-37°) the activity of limit dextrinase is relatively small, as the optimum for its activity is at 55-60°.

Special attention was paid to the removal of long glycosidic chains that were not decomposed by the malt enzymes. Even when present in very small amounts, they may form a substrate for α-1,4-glucosidase, and lead to erroneous results in estimations of dextrinolytic activity. These long chains could not be detected in the saccharified starch solution but the violet colour with iodine was clearly seen in the final product. This fraction formed a fluffy precipitate; its appearance was independent of the conditions of starch saccharification. Presumably this is undecomposed amylose that underwent retrogradation and is resistant to the action of enzymes. The removal of this fraction by centrifugation or by filtration appeared necessary. No colour with iodine was observed with the obtained preparations of dextrins even if they were in dry form.

By fractionated precipitation of dextrins with ethanol, a considerable part of long glycosidic chains was eliminated; thus the specificity of

the substrate was enhanced. The susceptibility of the obtained preparations to the action of malt α- and β-amylases was very low.

The differences in chemical properties of different dextrin preparations were very small, and were not accompanied by differences in biochemical properties. It should be pointed out that on addition of KH_2PO_4 (according to Pronin [4]) to the solution of limit dextrins the pH values differ in successive preparations and affect the final results of dextrinolytic activity measurements. On the other hand, when the pH was adjusted electrometrically, the differences were but slight and they could be ascribed only to structure of dextrins.

The author expresses his gratitude to Professor Dr. F. Nowotny for valuable suggestions and advice given in the course of this work.

SUMMARY

A modified procedure for preparation of limit dextrin substrate was elaborated. Starch paste was solubilized under pressure and saccharified with 7-day-old brewer's malt at 37°. The retrograded fractions were removed by filtration, and fermentable sugars by incubation with compressed yeast. The deproteinized solution was concentrated and fractionated with ethanol, the limit dextrins being precipitated by 58-80% ethanol concn. The yield was 3-4%, the content of glucose after hydrolysis 85-90%, average chain length 7-8 glucose units, and total phosphorus 0.2-0.25%. The obtained product was a suitable substrate for estimating the dextrinolytic activity of malt and *Aspergillus oryzae* extracts.

REFERENCES

[1] Klimowski D. N. & Rodzewicz W. I. - *Mikrobiołogija* **19**, 60, 1950.

[2] Müller E. - *Z. physiol. Chem.* **237**, 35, 1935.

[3] Pawlowski-Doemens, *Die brautechnischen Untersuchungsmethoden*, p. 156, R. Oldenbourg Verl., München - Berlin 1938.

[4] Pronin S. I., *Amiłoliticzeskije fermienty i ich rol w piszczewoj promyszlennosti*, s. 166-8, GIZLEPISZCZEPROM, Moskwa 1953.

[5] Redfern S. - *Wallerstein Lab. Comm.* **13**, 41, 89, 1950.

[6] Schwarz G. & Malsch L. - *Die Branntweinwirtschaft* **76**, 278, 1954.

[7] Schwarz G. & Malsch L. - *Die Branntweinwirtschaft* **76**, 299, 1954.

[8] Winton A. L. & Winton K. B., *The analysis of foods*, p. 191, J. Wiley & Sons. Chapman Hall Ltd., N.Y., London 1947.

ZMODYFIKOWANA METODA OTRZYMYWANIA DEKSTRYN GRANICZNYCH PRZY UŻYCIU SŁODU JĘCZMIENNEGO

Streszczenie

Opracowano zmodyfikowaną metodę otrzymywania dekstryn granicznych. Upłynniony pod ciśnieniem kleik skrobiowy scukrza się 7-dniowym słodem w temp. 37°, usuwa osad frakcji zretrogradowanych i odfermentowuje stosując drożdże piekarnicze. Po wytrąceniu białka płyn zagęszcza się i frakcjonuje etanolem. Dekstryny graniczne wytrącają się w 58-80% etanolu. Wydajność procesu 3-4%, zawartość glikozy po hydrolizie 85-90%, średnia długość łańcucha 7-8 reszt glikozowych, fosfor całkowity 0.2-0.25%. Otrzymany produkt jest odpowiednim substratem dla oznaczania aktywności dekstrynolitycznej słodu i ekstraktu z *Aspergillus oryzae*.

Received 22 April 1963.

ACTA BIOCHIMICA POLONICA

Vol. XI 1964 No. 1

T. CHOJNACKI

ON THE *IN VITRO* FORMATION OF PHOSPHOLIPIDS CONTAINING UNNATURAL BASES, *VIA* THE CYTIDINE MECHANISM

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa

It was shown previously [2, 3] that the enzymic system of lecithin and cephalin synthesis from CMP-PC [1] and CMP-PE respectively, could also be responsible for the formation of *N*-monomethyl and *N,N*-dimethylcephalins if the respective cytidine diphosphate bases were added to the homogenates of rat brain and liver tissues. Also diethylaminoethanol phosphate may follow this pathway giving rise to an unnatural phospholipid [9]. It should be mentioned that while a replacement of choline in cytidine diphosphate choline by another structurally related compound is possible, no other nucleotide base could replace the cytosine in the nucleotide without a complete loss of the precursor activity of the compound [18, 10].

The aim of the present work was to check whether other phosphorylated aminoalcohols structurally related to choline and ethanolamine can form phospholipids *via* the cytidine mechanism. This study was performed on four bases, 1-aminopropan-2-ol (AiPr), 2-amino-2-methylpropan-1-ol (AMPr), 3-dimethylaminopropan-1-ol (DMAPr) and 2-guanidinoethanol (GE). 1-Aminopropan-2-ol might be considered as a normal constituent of the living organism due to its presence in the vitamin B_{12}, but in relation to phospholipids it can be considered as an unnatural component. As there may be a closer relation of some of these four bases to those previously tested [2, 3, 9] than to choline or ethanolamine, some of the experiments on 2-monomethylaminoethanol (MMAE), 2-diethylaminoethanol (DEAE), and 2-dimethylaminoethanol (DMAE) are also included in this paper.

---

[1] Abbreviations used: AiPr, 1-aminopropan-2-ol; AMPr, 2-amino-2-methylpropan-1-ol; C, choline; DEAE, 2-diethylaminoethanol; DMAE, 2-dimethylaminoethanol; DMAPr, 3-dimethylaminopropan-1-ol; E, ethanolamine; GE, 2-guanidinoethanol; MMAE, 2-monomethylaminoethanol. P indicates *O*-phosphoric esters of the above alcohols; CMP, cytidine phosphate.

MATERIALS AND METHODS

Animals. The rats 6 weeks old, the chickens 6 weeks to 3 months old and adult frogs *Rana temporaria* of both sexes were used.

Special reagents. CMP, Sigma, USA; dicyclohexylcarbodiimide, 2-monomethylaminoethanol and 2-dimethylaminoethanol, BDH, London; 2-amino-2-methylpropan-1-ol and 3-dimethylaminopropan-1-ol, L. Light & Co. Ltd. Colnbrook, England; D, L-1-aminopropan-2-ol was a gift from Prof. Dr. K. Bernhauer; *O*-methylisourea was a gift from Dr. Z. Piasek. ^{32}P-labelled orthophosphoric acid was of French origin; aluminum oxide, Brockman activity 2, was Savory & Moore (Great Britain) product.

Procedures. The incubation of tissue homogenates was made as described previously [9]. The assay of ^{32}P was performed with VA-Z 410 VEB Vacutronic liquid counter, phosphorus was estimated according to Strickland *et al.* [24]. The content of cytidine was estimated spectrophotometrically at 281 mμ on the basis of the $\varepsilon_{max} = 13.6$ for CMP in acid solution. Paper chromatography of phosphoric esters and nucleotides was performed after Ebel on Whatman no. 1 paper in a mixture of propan-2-ol, 75 ml., water 25 ml., trichloroacetic acid, 5 g., conc. ammonia ($d_{20} = 0.895$), 0.3 ml. [13]. The spots were located according to Hanes & Isherwood [15]. The autoradiograms of paper chromatograms were made using the "Foton" (Poland) X-ray plates (44 CUK). The lipids were extracted from the incubation mixture according to Folch *et al.* [14]. The column chromatography of phospholipids on the aluminum oxide was performed with chloroform - methanol (1:1, v/v) mixtures containing increasing concentrations of water according to Long & Staples [19] as previously described [3]. For studying the products of alkaline hydrolysis of the phospholipids, the mild alkaline hydrolysis of lipids extracted from the incubated sample containing 200 - 300 mg. of tissue was performed according to Dawson [12], the hydrolysate being freed from Na^+ ions by passing through a column of Amberlite IRC-50(H^+). Then the water-soluble phosphorus compounds (glycerylphosphorylcholine, glycerylphosphorylethanolamine and others) were separated by two-dimensional paper chromatography [11].

Chemical syntheses of labelled compounds

The synthesis of asymmetric pyrophosphoric diesters, P^1-cytidine, P^2-alcoholobase was performed by coupling the respective ^{32}P-labelled phosphoric esters with CMP in the presence of dicyclohexylcarbodiimide by the method of Kennedy [16]. The details of the isolation of the product from the reaction mixture were the same as described previously for CMP-^{32}PDEAE [9]. In some cases the crude product was separated

by paper chromatography on Whatman no. 1 paper in the acid solvent system of Ebel [13] and purified by column chromatography.

In Table 1 the molar ratios of cytidine to phosphorus of the obtained nucleotides are presented, as well as the ratios of their specific activities (counts/min./μg.P) to those of the respective ester. They are close to the theoretical values.

Table 1

Analysis of synthetic cytidine diphosphate bases

Nucleotide	Molar ratio cytidine:phosphorus		Ratio of specific activities CMP-Pbase:Pbase	
	calc.	found	calc.	found
CMP-^{32}PAiPr	1:2	1:2.04	0.5	0.49
CMP-^{32}PAMPr	1:2	1:1.91	0.5	0.54
CMP-^{32}PDMAPr	1:2	1:1.90	0.5	0.51
CMP-^{32}PGE	1:2	1:2.06	0.5	0.52

As revealed by paper chromatography (Fig. 1) the products were chromatographically pure, and on acid hydrolysis (1 N-HCl, 1 hr., 100°) they were decomposed with the formation of CMP and the labelled respective phosphoric esters. In the case of CMP-^{32}PAMPr the acid hydrolysis gave rise to the formation of two radioactive spots, one of the parent phosphoric ester and the other with the R_F corresponding to orthophosphate.

Cytidine diphosphate 1-aminopropan-2-ol (CMP-^{32}PAiPr). The *O*-phosphoryl ester of AiPr was prepared by heating under reduced pressure (oil pump) equimolar amounts of ^{32}P-labelled orthophosphoric acid (approx. 10 mg. P) and 1-aminopropan-2-ol in the presence of P_2O_5, kept in separate vessel, at 160 - 170° during 3 hr. The reaction mixture after cooling was dissolved in a few milliliters of water and hydrolysed on a boiling water bath for 15 min. The solution was adjusted to pH 8, diluted to the volume of 100 ml. and passed through the Dowex 1 formate column (10 × 1.2 cm.). The column was washed with 100 ml. of water and the ^{32}PAiPr eluted with 0.05 M-HCOOH. The yield of the synthesis was about 50%. The product (^{32}PAiPr) was chromatographically pure. Its phosphorus:nitrogen molar ratio was 1:1.07 (calculated 1:1).

For obtaining CMP-^{32}PAiPr, in a glass stoppered flask 20 mg. of CMP and an equimolar amount of ^{32}PAiPr were dissolved in the mixture of 2.33 ml. of pyridine and 0.33 ml. of 36% formaldehyde [17], then 0.6 g. of dicyclohexylcarbodiimide was added. The mixture was left at 37° for one week, two additional 0.4 g. portions of dicyclohexylcarbodiimide being added on the second and on the fourth day. The

semisolid reaction mixture was mixed with 10 ml. water, washed with ethyl ether, the aqueous phase after filtration was again washed twice with ether, applied as a band on a 30 cm. wide sheet of paper and chromatographed in the acid solvent after Ebel [13]. The radioactive

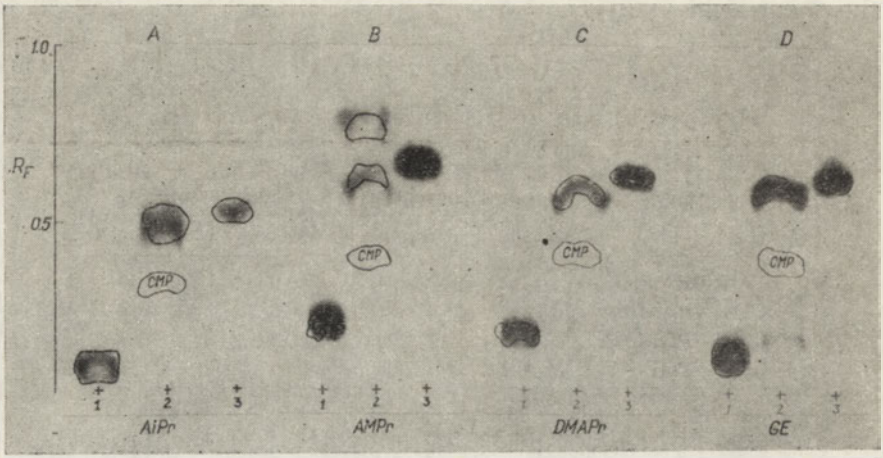


Fig. 1. Autoradiogram of paper chromatogram of synthetized CMP derivatives of ^{32}P-labelled phosphoric esters. (1), The labelled nucleotide synthetized; (2), the products of its hydrolysis (100°, 1 N-HCl, 1 hr.); (3), the phosphoric ester used for the synthesis of the nucleotide. The ascending technique was used on Whatman no. 1 paper in propan-2-ol, 75 ml.; water, 25 ml.; trichloroacetic acid, 5 g.; conc. ammonia ($d_{20} = 0.895$), 0.3 ml. [13]. (*A*), CMP-^{32}PAiPr; (*B*), CMP-^{32}PAMPr; (*C*), CMP-^{32}PDMAPr; (*D*), CMP-^{32}PGE. The phosphorus spots were marked with pencil.

compounds were located by autoradiography. The main radioactive band consisted of the unreacted ^{32}PAiPr (R_F 0.5). CMP-^{32}PAiPr gave a faint band with R_F about 0.1. This was cut off, washed with ethyl ether to remove the trichloroacetic acid of the solvent, and eluted with water. The eluate was adjusted to pH 8, diluted to 100 ml. and passed through the Dowex 1 formate column (10 × 1.2 cm.). The elution was made by linear concentration gradient of HCOOH using 300 ml. of water in the mixing chamber and 300 ml. of 0.04 M-HCOOH in the reservoir; 10 ml. fractions were collected. The radioactive peak exhibiting the absorption at 280 mμ was obtained between 110 and 140 ml. of the effluent. This fraction was evaporated to dryness at room temperature, dissolved in 2 ml. of water, adjusted to pH 7, and centrifuged to remove the fine particles of the resin. On paper chromatography traces of radioactive impurities (not visible in the Fig. 1*A*) besides the main radioactive, UV absorbing spot were found. The specific activity of CMP-^{32}PAiPr used in the experiments with tissue homogenates was 58 000 counts/ /min./μmole.

Cytidine diphosphate 2-amino-2-methylpropan-1-ol (CMP-^{32}PAMPr). The *O*-phosphoryl ester was prepared from AMPr and ^{32}P-labelled orthophosphoric acid as described above for ^{32}PAiPr. ^{32}PAMPr obtained from the Dowex 1 formate column eluate showed the phosphorus:nitrogen molar ratio of 1:1.09 (calculated, 1:1). For the synthesis of the nucleotide, 10 mg. of CMP and an equimolar amount of ^{32}PAMPr were dissolved in the mixture of 1.4 ml. of pyridine and 0.2 ml. of water, and 0.45 g. of dicyclohexylcarbodiimide was added to the mixture. This was left for one week at 37°, two additional portions of 0.3 g. of dicyclohexylcarbodiimide being added on the second and on the fourth day.

The isolation of CMP-^{32}PAMPr from the reaction mixture consisted of paper and subsequent column chromatography performed in the same manner as for CMP-^{32}PAiPr. The specific activity of CMP-^{32}PAMPr used in the experiments with tissue homogenates was 37 500 counts/ /min./μmole.

Cytidine diphosphate 3-dimethylaminopropan-1-ol (CMP-^{32}PDMAPr). The *O*-phosphoryl ester was obtained by heating equimolar amounts of ^{32}P-labelled orthophosphoric acid and DMAPr as in the Riley's procedure for obtaining phosphorylcholine [23]. Only about 15% of the phosphate present in the reaction mixture underwent esterification. The reaction mixture after 15 min. hydrolysis at 100° was passed through the column of Amberlite IR 120 (NH_4^+ form). The effluent was adjusted to pH 8 and passed through the column (10 × 1.2 cm.) of Amberlite IRA 400 Cl^-. The effluent and subsequent washings contained the phosphoric ester which was chromatographically pure. In different solvent systems it was identical with the standard preparation of PDMAPr synthetized in this laboratory from DMAPr and $POCl_3$. The ^{32}PDMAPr was converted into the pyridinium salt by passing through the column of Dowex 50 pyridinium form. The details of the coupling with CMP and the procedure of the isolation of the product were identical with those for the preparation of CMP-^{32}PDEAE [9]. The specific activity of CMP-^{32}PDMAPr used in the experiments with tissue homogenates was 39 000 counts/min./μmole.

Cytidine diphosphate guanidinoethanol (CMP-^{32}PGE). The *O*-phosphoryl ester of guanidinoethanol was prepared according to Beatty & Magrath [6] by the action of *O*-methylisourea on the ^{32}P-labelled phosphorylethanolamine obtained according to Artom [5]. The alkaline reaction mixture was diluted and freed from Na^+ ions by passing through a Dowex 1 formate column. All the radioactivity remained on the resin. The attempt to isolate pure ^{32}PGE by elution with increasing concentrations of HCOOH was unsuccessful, as the ^{32}PGE fraction was still accompanied by the traces of the unreacted ^{32}PE. Therefore the eluted radioactive fraction was submitted to preparative paper chromatography with the acid solvent system of Ebel [13] and a good separation

of ^{32}PGE (R_F 0.67) from the traces of ^{32}PE (R_F 0.45) was achieved. The band of ^{32}PGE was cut off, washed with ether, eluted with water, and the ammonium salt of ^{32}PGE was converted into the acid form by the absorption on, and elution from, the Dowex 1 formate column. The obtained product had the phosphorus:nitrogen molar ratio of 1:2.79 (calculated 1:3). It moved as single spot in a variety of solvents on paper, and gave a positive reaction on the chromatogram with the modified Sakaguchi reagent [1].

For obtaining CMP-^{32}PGE, 5 mg. of CMP and an equimolar amount of ^{32}PGE were dissolved in the mixture of 0.7 ml. of pyridine and 0.1 ml. of water, and 150 mg. of dicyclohexylcarbodiimide was added. The mixture was left at 37° for one week, two additional 90 mg. portions of dicyclohexylcarbodiimide being added on the second and on the fourth day. The isolation of CMP-^{32}PGE from the reaction mixture was performed as above in the case of the first two nucleotides. The final radioactive, UV absorbing product gave also a positive reaction on paper chromatogram with the modified Sakaguchi reagent [1]. The specific activity of CMP-^{32}PGE used in the experiments with tissue homogenates was 50 000 counts/min./μmole.

Other labelled cytidine diphosphate bases. Cytidine diphosphate ethanolamine (CMP-^{32}PE), cytidine diphosphate 2-monomethylaminoethanol (CMP-^{32}PMMAE), cytidine diphosphate 2-dimethylaminoethanol (CMP-^{32}PDMAE), cytidine diphosphate 2-diethylaminoethanol (CMP-^{32}PDEAE), and cytidine diphosphate choline (CMP-^{32}PC) were synthetized by coupling the CMP with the appropriate *O*-phosphoryl esters according to Kennedy [16] as described previously [3, 2, 9].

The efficiency of the reaction of coupling the CMP with phosphoric esters in the presence of dicyclohexylcarbodiimide in the procedure of Kennedy [16] largely depends on the basic character of the phosphoester component. The highest yields were obtained for CMP-PGE (up to 60%), CMP-PC (30 - 40%), CMP-PDEAE (20 - 30%), CMP-PDMAE (20 - 30%), CMP-PDMAPr (30%), CMP-PMMAE (about 20%), while for CMP-PE, CMP-PAMPr and CMP-PAiPr the yields usually did not exceed 5% and were not improved by addition of formaldehyde instead of water [17] to the reaction mixture.

RESULTS

In Table 2 the rates of incorporation of the labelled phosphoric esters from their cytidylyl derivatives into phospholipids are presented. In all the tissues tested the rate of ^{32}P incorporation was considerable no matter what kind of base was present in the original molecule of cytidine nucleotide. It seems highly improbable that any ^{32}P incorporation could occur *via* the free phosphate, even if a complete cleavage of the labelled nucleotide took place in the incubation mixture.

Table 2

The labelling of phospholipids on incubation of tissue homogenates with cytidylyl derivatives of different ^{32}P-labelled phosphoric esters

Each incubation sample contained 100 mg. of homogenized tissue in a medium containing: 31.6 mM-KCl; 9.5 mM-NaF; 20 mM-$MgCl_2$; 4 mM-Na_2HPO_4; 26.6 mM-tris-HCl, pH 7.4; and 0.05 μmole of the respective labelled nucleotide. Final volume of the incubate was 1.5 ml. Incubation, 1 hr. at 37°. The phospholipids were extracted according to Folch *et al.* [14] and the radioactivity was measured as described under Methods.

$$\text{Rel. act.} = \frac{\text{counts/min./μmole of lipid P}}{\text{counts/min./μmole of cytidylyl precursor}} \times 1000$$

Compound	Chicken liver		Rat brain		Rat liver		Frog liver	
	^{32}P incorp. (%)	Rel. act.	^{32}P incorp. (%)	Rel. act.	^{32}P incorp. (%)	Rel. act.	^{32}P incorp. (%)	Rel. act.
CMP-^{32}PE	30.1	6.10	19.7	2.84	28.5	6.54		
	28.1	6.54	20.7	3.02	25.3	6.75		
	30.6	7.44	17.6	1.52	20.1	3.45		
			25.0	3.37	18.8	2.58		
			18.0	2.11	16.8	3.21		
CMP-^{32}PMMAE	39.9	10.3	17.4	2.44	22.9	4.57	31.8	9.10
	39.2	9.44	17.9	2.15	20.6	5.47	29.5	8.45
			22.1	3.34	18.0	3.06		
			21.5	3.12	13.9	3.11		
CMP-^{32}PDMAE	48.5	11.5	36.9	3.74	45.9	9.03	61.3	14.2
	47.9	11.3	39.0	3.96	44.1	8.88	57.9	14.1
			43.2	5.11	36.3	5.69		
			33.5	5.79				
CMP-^{32}PC	48.0	7.60	46.2	5.00	58.4	7.50	67.5	20.9
	53.5	9.30	29.2	2.60	46.6	7.70	42.6	12.4
			33.2	3.00	40.1	7.00	51.7	14.5
			23.8	3.97	40.0	6.82		
CMP-^{32}PDEAE*	58.5	10.2	16.1	2.30	23.6	3.95		
CMP-^{32}PAiPr	17.6	3.42	8.7	1.30	11.4	2.66		
	16.2	3.77	7.1	1.25	9.1	2.12		
			8.3	1.46	10.8	2.10		
			6.1	1.26	10.0	1.77		
CMP-^{32}PAMPr	57.5	12.2	8.9	1.00	14.7	2.52	22.3	5.90
	40.2	5.82	18.5	1.68	8.1	1.19		
	46.0	6.80	21.8	2.98	8.7	1.25		
CMP-^{32}PDMAPr	50.5	10.4	10.9	1.25	15.8	2.53	25.0	5.90
	40.5	7.16	14.6	1.70	11.6	1.67		
	42.7	7.73	22.5	2.64	12.0	1.75		
CMP-^{32}PGE	50.0	9.20	10.2	1.25	19.3	3.59	27.4	6.87
	35.6	6.26	9.5	1.30	14.4	2.22		
	21.7	6.58	17.3	1.92	17.3	2.73		

* Data from [9].

However, some differences in the incorporation were observed; thus the two natural compounds, CMP-PC and CMP-PE were not equally effective, the incorporation of PC being twice as high as that of PE.

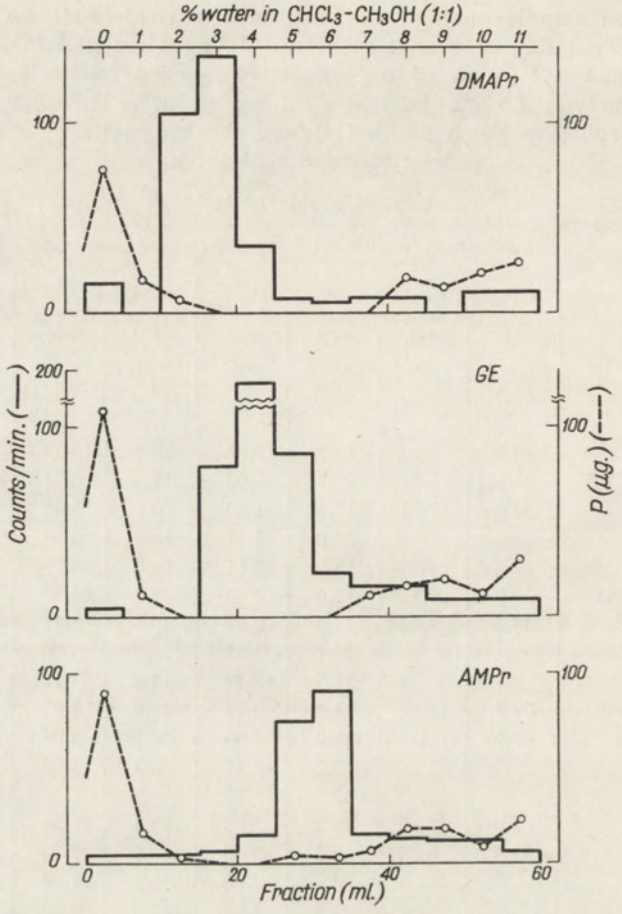


Fig. 2. Chromatography on aluminum oxide of the lipid extracts obtained after the incubation of rat liver homogenate with CMP-^{32}PDMAPr, CMP-^{32}PAMPr and CMP-^{32}PGE. An amount of lipid corresponding to 0.3 g. of fresh tissue was applied to a column (0.7 × 3.0 cm.) filled with 2 g. of aluminum oxide, and the elution carried out in a step-wise fashion. (- - ○ - -), Phosphorus content; (——) radioactivity.

The incorporation of PMMAE was similar to that of PE, and of PDMAE to PC. The differences in the incorporation of five other phosphorylated bases were dependent on the tissue tested.

The highest rates of the incorporation for all the nucleotides tested were obtained with the chicken liver homogenates. The incorporation of 2-amino-2-methylpropan-1-ol (AMPr), 2-guanidinoethanol (GE) and 3-dimethylaminopropan-1-ol phosphates did not seem to be lower when compared with the incorporation of ^{32}PE and ^{32}PDMAE. The incorpora-

tion of ^{32}PAiPr was, however, lower than the incorporation of ^{32}PE. In rat liver the rate of incorporation of ^{32}PAiPr, ^{32}PAMPr, ^{32}PDMAPr and ^{32}PGE was lower than the incorporation of ^{32}PE and its three *N*-methylated derivatives. This is true for the rat brain cortex as well. In the frog liver no experiment was made with CMP-^{32}PE, and no proper comparison for ^{32}PAMPr can therefore be made; however, the incorporation of this and ^{32}PGE and ^{32}PDMAPr was lower (approx. by half) than that of ^{32}PC and ^{32}PDMAPr. When the lipid extracts containing the radioactive phospholipids formed in tissue homogenates from labelled CMP-PAMPr, CMP-PDMAPr and CMP-PGE were chromatographed on the aluminum oxide column [19, 3] their elution patterns were different from those of the natural phospholipids present in the lipid extract (Fig. 2). However, neither by this method, nor on silica paper chromatography according to Marinetti [21], the phospholipid formed from CMP-^{32}PAiPr could be separated from the natural cephalins.

The products of the mild alkaline hydrolysis [12] of phospholipids formed from ^{32}P-labelled CMP-PAiPr, CMP-PAMPr, and CMP-PGE

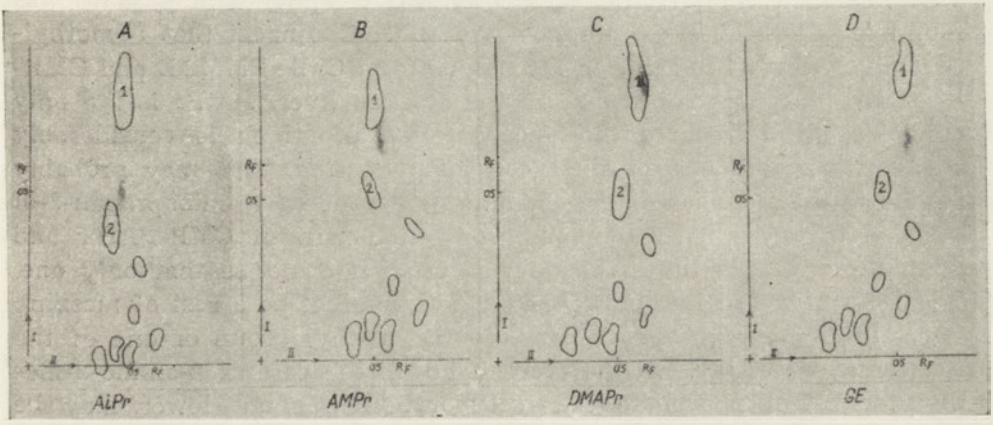


Fig. 3. Autoradiograms of two-dimensional chromatograms of alkaline hydrolysates of the phospholipids of rat brain homogenates incubated with: (*A*), CMP-^{32}PAiPr; (*B*), CMP-^{32}PAMPr; (*C*), CMP-^{32}PDMAPr; (*D*), CMP-^{32}PGE. Details of incubation as in Table 2. The following solvent systems were employed: *I*, phenol - water - conc. ammonia, $d_{20} = 0.895$ (80:20:0.3, w/v/v/), descending technique; *II*, methylpropan-2-ol (62:38, v/v) - trichloroacetic acid (10%, w/v), ascending technique [11]. The dark areas represent the radioactive material. The phosphorus containing spots, (1), glycerylphosphorylcholine, (2), glycerylphosphorylethanolamine, and others were localized with the acid molybdate spray [15] and marked with pencil.

had in two-dimensional chromatography different R_F values from natural phospholipid derivatives (Fig. 3). However, the hydrolysis product of the phospholipid formed from CMP-PDMAPr could not be separated from glycerylphosphorylcholine in this system.

An additional proof that a guanidinophospholipid was formed from CMP-^{32}PGE in tissue homogenate was provided by the demonstration that the respective mild alkaline hydrolysis product of the labelled phospholipid could easily be detected by spraying the chromatogram with the modified Sakaguchi reagent.

DISCUSSION

It was shown in this study that a variety of phosphorylated aminoalcohols might be incorporated into phospholipids from their cytidine diphosphate compounds. This would mean that the specificity of enzyme(s) transferring the phosphorylcholine and phosphorylethanolamine onto the diglycerides, is very low. There seem also to exist differences in specificity of enzymes from different sources.

The enzyme system from chicken liver is known as a very active one, and was used in the majority of studies on the biosynthesis of lecithin [18]. In this paper it was found that in the homogenate of chicken liver PAMPr, PDMAPr and PGE were incorporated into phospholipids at a similar rate as the natural compounds; the same was found for PDEAE. The finding of Ansell & Chojnacki that *N*-methylcephalins were synthetized in rat tissues from CMP-PMMAE and CMP--PDMAE [3, 2] was confirmed on the chicken liver. PAiPr is the only aminoalcohol phosphate the incorporation of which is considerably smaller than that of the other compounds tested. This may probably be due to the presence of the asymmetric carbon of 1-aminopropan-2-ol close to the pyrophosphate bond in the molecule of CMP-PAiPr and as in these studies the racemate was used, it could be that only one, D or L form was in fact incorporated. There might be a sort of masking effect of the pyrophosphate bond by the methyl group of one of the steric forms of AiPr. On the other hand, in the case of 2-amino-2-methylpropan-1-ol phosphate the incorporation was not lower may be because of the primary hydroxyl group being esterified and the methyl groups being not so close to the pyrophosphate bond. There could be an analogy with the lipolytic enzymes, which, as established by Bergström *et al.* [7], do not act on the glycerol ester bond of the fatty acid containing methyl groups on $C_{(2)}$. The elongation of the carbon chain by one (DMAPr) had no effect on the rate of incorporation when compared with DMAE, similarly as the replacement of the amino group by the guanidinic one.

In rat and frog tissues the rates of incorporation of PAiPr, PAMPr, PDMAPr and PGE were smaller than those observed with PE and its *N*-methyl derivatives. This was true also for PDEAE [9].

The high rate of incorporation of unnatural phosphobases in chicken liver might be ascribed to the generally faster process of phosphoric

ester incorporation in this tissue which could mask any initial differences, as the labelling of phospholipids was measured after 1 hr. of incubation. However, in the frog liver, where the ^{32}PC was incorporated at the same rate as in the chicken liver, the labelling from the unnatural phosphorylated aminoalcohols was smaller. It may also be that there are differences in the fatty acid composition of diglycerides in the tissues studied, the diglycerides from chicken liver being of lower specificity as acceptors for phosphoric esters, than those of rat and frog.

The relative amount of the unnatural phospholipid formed in the homogenate depends not only on the rate of incorporation of the respective phosphorylated base, but also on the amount of tissue phospholipids present in the homogenate. The values of relative activity (rel. act.) cited in Table 2 denote the proportion of the unnatural phospholipid per 1000 molecules of endogenous phospholipid. This proportion was rather low for AiPr but could be raised even up to 50 in the experiments where the chicken liver mitochondrial fraction was incubated for 3 hr., with fresh portions of CMP-^{32}PAiPr being added every 30 min.

It might be expected that better yields of several unnatural phospholipids could be obtained if the diglyceride acceptor of phosphorylated aminoalcohols would be added to the incubation medium. It is hoped that this type of biosynthesis would furnish an alternative method for preparing phospholipids, to the chemical ones.

There are in the literature several examples for the incorporation of different free unnatural aminoalcohols into phospholipids, based on the *in vivo* experiments [22, 20, 8]. One of them 2-amino-2-methylpropan--1-ol [20] was found in the present experiments to be incorporated as phosphoric ester into phospholipids from its cytidylyl derivative. The specificity of the respective transferase appears to be very low, and this allows to introduce unnatural bases into phospholipids. However, it has to be found whether natural enzyme(s) can phosphorylate unnatural aminoalcohols and convert them into cytidylyl derivatives. In the case of 2-dimethylaminoethanol its phosphorylation in brain tissue was demonstrated by Ansell & Spanner [4] and it was found that PDMAE does react with CTP (Ansell & Chojnacki, unpublished results). If so, the kind and the proportion of the nitrogenous base in the tissue glycerophosphatides would be only the reflection of the occurrence of these compounds in the cell. Therefore the possibility of biosynthesis of abnormal phospholipids should be seriously considered in all the conditions when a substance structurally related to choline or ethanolamine is introduced or may be formed in the organism. Since the phospholipids are essential constituents of cell enzyme systems, any change in their chemical composition by introducing a "false" element might modify the activity of these enzyme systems.

The author wishes to thank Prof. Dr. T. Korzybski from the Institute of Biochemistry and Biophysics in Warsaw for advice and encouragement during these experiments. Thanks are also due to Dr. G. B. Ansell from the Department of Experimental Neuropharmacology, Birmingham University, England, for helpful discussion and for providing some chemicals. To Prof. Dr. K. Bernhauer (Technische Hochschule, Stuttgart, Germany), Prof. Dr. T. Urbański and Dr. Z. Piasek (Institute of Organic Synthesis, Polish Academy of Sciences, Warsaw) and Dr. W. Wieniawski (Institute of Drugs, Warsaw) the author is grateful for gifts of, respectively, D,L-threamine, *O*-methylisourea and 3-dimethylaminopropan-1-ol.

SUMMARY

Four unnatural analogues of cytidine diphosphate choline containing 1-aminopropan-2-ol, 2-amino-2-methylpropan-1-ol, 3-dimethylaminopropan-1-ol and 2-guanidinoethanol instead of choline, were chemically synthetized from CMP and the respective *O*-phosphoric esters of these unnatural bases. The unnatural cytidine coenzymes served as precursors in phospholipid biosynthesis in the chicken, rat and frog tissues. The phospholipids formed from them can be distinguished from the normal phospholipids by means of chromatographic procedures.

REFERENCES

[1] Acher R. & Crocker C. - *Biochim. Biophys. Acta* **9**, 704, 1952.
[2] Ansell G. B. & Chojnacki T. - *Biochem. J.* **85**, 31P, 1962.
[3] Ansell G. B. & Chojnacki T. - *Nature* **196**, 545, 1962.
[4] Ansell G. B. & Spanner S. - *J. Neurochem.* **9**, 253, 1962.
[5] Artom C., in *Methods in Enzymology* (Eds. S. P. Colowick & N. O. Kaplan) vol. **4**, p. 815, Academic Press, New York, 1957.
[6] Beatty I. M. & Magrath D. J. - *Nature* **183**, 591, 1951.
[7] Bergström S., Borgström B., Tryding N. & Westöö G. - *Biochem. J.* **58**, 604, 1954.
[8] Bieber L. L., Brookes V. J., Cheldelin V. H. & Newburg R. W. - *Biochem. Biophys. Res. Com.* **6**, 237, 1961.
[9] Chojnacki T. & Korzybski T. - *Acta Biochim. Polon.* **10**, 233, 1963.
[10] Chojnacki T. & Korzybski T. - *Acta Biochim. Polon.* **10**, 455, 1963.
[11] Dawson R.M.C. - *Biochim. Biophys. Acta* **14**, 374, 1954.
[12] Dawson R. M. C. - *Biochem. J.* **75**, 45, 1960.
[13] Ebel J. P. - *Bull. Soc. Chim. Biol.* **34**, 321, 1952.
[14] Folch J., Lees M. & Sloane-Stanley G. H. - *J. Biol. Chem.* **226**, 497, 1957.
[15] Hanes C. S. & Isherwood F. A. - *Nature* **164**, 1107, 1949.
[16] Kennedy E. P. - *J. Biol. Chem.* **222**, 185, 1956.
[17] Kennedy E. P., Borkenhagen L. F. & Wagner-Smith S. - *J. Biol. Chem.* **234**, 1998, 1958.
[18] Kennedy E. P. & Weiss S. B. - *J. Biol. Chem.* **222**, 193, 1956.
[19] Long C. & Staples D. A. - *Biochem. J.* **80**, 557, 1961.

[20] Longmore W. J. & Mulford D. J. - *Biochem. Biophys. Res. Com.* **3**, 556, 1960.
[21] Marinetti G. V. - *J. Lipid. Res.* **3**, 1, 1962.
[22] McArthur C. S., Lucas C. C. & Best C. H. - *Biochem. J.* **41**, 612, 1947.
[23] Riley R. F. - *J. Am. Chem. Soc.* **66**, 512, 1944.
[24] Strickland K. P., Thompson R. H. S. & Webster G. R. - *J. Neurol. Neurosurg. Psychiat.* **19**, 12, 1956.

O POWSTAWANIU *IN VITRO* DROGĄ MECHANIZMU CYTYDYNOWEGO FOSFOLIPIDÓW ZAWIERAJĄCYCH NIENATURALNE ZASADY

Streszczenie

Zsyntetyzowano chemicznie cztery analogi cytydynodwufosfocholiny zawierające zamiast choliny, 1-aminopropan-2-ol, 2-amino-2-metylopropan-1-ol, 3-dwumetyloaminopropan-1-ol i 2-gwanidynoetanol. Do syntez użyto CMP oraz estrów *O*-fosforowych powyższych nienaturalnych zasad. Uzyskane nienaturalne koenzymy cytydynowe spełniały rolę prekursorów fosfolipidów w homogenatach tkanek kurczęcia, szczura i żaby. Wytworzone z nich fosfolipidy można oddzielić od fosfolipidów naturalnych metodami chromatograficznymi.

Received 9 July 1963.

ACTA BIOCHIMICA POLONICA

Vol. XI 1964 No. 1

L. ŻELEWSKI

CITRATE SYNTHESIS IN THE KIDNEY AND LIVER OF RATS TREATED WITH NICOTINAMIDE AND OESTRADIOL BENZOATE

Department of Biochemistry, Medical School, Gdańsk

It has been found in this laboratory that the administration of large doses of oestradiol benzoate to female rats was followed by a decrease of citrate excretion in the urine [8]. Further experiments [7] have shown that the coupled administration of oestradiol benzoate and nicotinamide caused an increase in citrate and α-ketoacids excretion on the first day after the injection, followed by a decrease on the second and third day.

The aim of the present work was to study the changes in the rate of citrate synthesis in the kidney and liver of rats which had been injected with nicotinamide and oestradiol benzoate, and to compare the results with changes in citrate excretion.

MATERIALS AND METHODS

Animals. White litter-mate female rats aged four months were used; they were fed the standard diet [9] for at least a fortnight before the experiment. Then they were injected intramuscularly with single or double doses of 10 mg. per kg. body weight of oestradiol benzoate dissolved in arachis oil and/or single or double doses of 500 mg. per kg. body weight of nicotinamide, administered intraperitoneally. The animals were killed by decapitation at specified time intervals after the first injection.

Analytical procedures. The kidneys and liver were homogenized in a Potter-Elvehjem homogenizer with 7 volumes of KCl-borate solution (0.1 M-KCl buffered with 0.039 M-borate, pH 7.0). One ml. of the homogenate was incubated with 2 ml. of a freshly made solution containing 30 μmoles oxaloacetate, 60 μmoles pyruvate, 15 μmoles fluoroacetate, 90 μmoles sodium phosphate and 15 μmoles $MgCl_2$. The incubation was carried out in Warburg flasks at 30° and was stopped by the addition of 0.5 ml. of 40% trichloroacetic acid. The protein precipitate was centrifuged off and citrate was estimated in the supernatant fluid [1].

The nitrogen content of the homogenates was estimated by the Kjeldahl procedure in a Parnas-Wagner apparatus using boric acid for the binding of ammonia.

Blood plasma was analysed for α-ketoacids by the method of Friedmann & Haugen [2] and for chloride content by the method of Van Slyke [3].

Chemicals. Oestradiol benzoate was a gift from Hoechst A.G., Frankfurt am Main (Germany). Oxaloacetic acid was obtained from the Department of Organic Chemistry, Technical High School in Gdańsk. Pyruvic acid was purchased from the Xenon (Łódź, Poland); 2,4-dinitrophenylhydrazine and fluoroacetate were obtained from L. Light & Co. (England), and the arachis oil from Warszawskie Zakłady Farmaceutyczne (Warszawa, Poland). Other chemicals were products of Fabryka Odczynników Chemicznych (Gliwice, Poland).

RESULTS

Four litters of four female rats each were taken for the first experiment. One rat from every litter served as control; to the other three, oestradiol benzoate and nicotinamide were administered as described in Table 1. The animals were killed and the rate of citrate synthesis cata-

Table 1

Citrate synthesis catalysed by the kidney homogenates of female rats at pH 7.0

Treatment: group I, 10 mg. oestradiol benzoate intramuscularly and 500 mg. nicotinamide intraperitoneally, per kg. body weight; group II and III, 10 mg. oestradiol benzoate and twice 500 mg. nicotinamide per kg. body weight, the second injection of nicotinamide being given on the next day. The animals were killed 13, 36 and 60 hr., respectively, after the first injection, as indicated in parentheses in the Table. For the composition of the incubation medium see text.

Mean values from 4 experiments are given, expressed as μmoles citrate per mg. of nitrogen content in the sample, ± S.D.

Incubation time (min.)	Control (non-treated)	Group I (13 hr.)	Group II (36 hr.)	Group III (60 hr.)
2	0.15 ± 0.01	0.20	0.26 ± 0.01	0.18
5	0.20 ± 0.02	0.34 ± 0.05	0.33 ± 0.02	0.24 ± 0.01
10	0.26 ± 0.04	0.39 ± 0.05	0.42 ± 0.04	0.32 ± 0.04
15	0.26 ± 0.01	0.43 ± 0.07	0.42 ± 0.04	0.32 ± 0.03
25	0.26 ± 0.03	0.43 ± 0.08	0.43 ± 0.03	0.33 ± 0.01

lysed by the kidney and liver homogenates was followed at pH 7.0 (Table 1). The synthesis was increased in kidney homogenates derived from rats which had been given oestradiol benzoate and nicotinamide

and were killed 13 hr. after the first injection, i.e. at the time of increased citrate excretion ($P = 0.01$). A similar increase in the rate of citrate synthesis was observed in the kidney homogenates of rats killed 36 and 60 hr. after the first injection ($P = 0.01$) although at that time *in vivo* a significant decrease in citrate and α-ketoacids excretion is observed [7].

For the second experiment four litters of four rats each were treated in the same way. These animals were also used for the determination of α-ketoacids and chloride levels in the blood plasma. The synthesis of citrate from pyruvate and oxaloacetate was followed at pH 7.8, i.e. the optimum pH for the citrate condensing enzyme (Table 2). Therefore the rate of citrate synthesis in the kidney homogenates was higher than in homogenates incubated at pH 7.0. The increase in the rate of citrate synthesis after oestradiol benzoate and nicotinamide injections was observed both in kidney and liver homogenates, the increase for the kidney being more pronounced.

Table 2

Citrate synthesis catalysed by the kidney and liver homogenates of female rats at pH 7.8

The animals were treated and killed as described in Table 1. For the composition of the incubation medium see text.
The mean values from 4 experiments are given expressed as μmoles citrate per mg. of nitrogen content in the sample, ± S.D.

Homogenate	Incubation time (min.)	Control (non-treated)	Group I (13 hr.)	Group II (36 hr.)	Group III (60 hr.)
Kidney	2	0.23 ± 0.01	0.23 ± 0.06	0.34 ± 0.05	0.31 ± 0.05
	5	0.43 ± 0.02	0.36 ± 0.04	0.61 ± 0.01	0.49 ± 0.05
	10	0.48 ± 0.14	0.64 ± 0.02	0.87 ± 0.18	0.69 ± 0.04
	15	0.50 ± 0.14	0.85 ± 0.14	1.12 ± 0.11	0.76 ± 0.17
	25	0.51 ± 0.08	0.79 ± 0.01	1.15 ± 0.23	0.68 ± 0.04
Liver	5	0.47 ± 0.06	0.45 ± 0.09	0.57 ± 0.08	0.49 ± 0.17
	10	0.82 ± 0.13	0.85 ± 0.08	1.04 ± 0.06	1.40 ± 0.23
	15	1.07 ± 0.02	1.05 ± 0.11	1.48 ± 0.21	1.40 ± 0.43
	25	1.69 ± 0.12	1.77 ± 0.23	2.11 ± 0.18	2.13

For the third experiment two litters of four rats each were treated with oestradiol benzoate and nicotinamide in the same way as for the first experiment. However, the incubation medium for citrate synthesis was of pH 7.4, i.e. the optimum pH for the decarboxylation of pyruvate (Table 3). Also here the amount of citrate synthetized by the kidney and liver homogenates was higher in animals which had been injected with oestradiol benzoate and nicotinamide.

Table 3

Citrate synthesis catalysed by the kidney and liver homogenates of female rats at pH 7.4

The rats were treated and killed as described in Table 1. For the composition of the incubation medium see text.

The mean values from 4 experiments are given expressed as μmoles citrate per mg. of nitrogen content in the sample, ± S.D.

Homogenate	Incubation time (min.)	Control (non-treated)	Group I (13 hr.)	Group II (36 hr.)	Group III (60 hr.)
Kidney	2	0.36 ± 0.03	0.41 ± 0.05	0.28 ± 0.01	0.31 ± 0.07
	5	0.49 ± 0.08	0.70 ± 0.06	0.65 ± 0.06	0.67 ± 0.08
	10	0.86 ± 0.01	0.92 ± 0.14	1.01 ± 0.17	1.15 ± 0.20
	15	0.95 ± 0.06	1.19 ± 0.08	1.32 ± 0.01	1.34 ± 0.27
	25	1.04 ± 0.01	1.28 ± 0.14	1.43 ± 0.23	1.42 ± 0.29
Liver	5	0.59 ± 0.07	0.73 ± 0.13	0.71 ± 0.14	0.71 ± 0.14
	10	0.89 ± 0.06	1.00 ± 0.06	1.00 ± 0.06	1.18 ± 0.05
	15	1.04 ± 0.27	1.43 ± 0.25	1.49 ± 0.19	1.86 ± 0.31
	25	1.51 ± 0.45	1.96 ± 0.31	2.00 ± 0.28	2.31 ± 0.30

Table 4

The effect of oestradiol benzoate and nicotinamide on citrate synthesis catalysed by kidney and liver homogenates of female rats

Treatment: group I, 10 mg. oestradiol benzoate per kg. body weight intramuscularly and two doses of 500 mg. each of nicotinamide per kg. body weight administered intraperitoneally on two successive days; group II, two doses of 500 mg. each of nicotinamide per kg. body weight on two successive days; group III, 10 mg. oestradiol benzoate per kg. body weight; group IV, two doses of 10 mg. each of oestradiol benzoate per kg. body weight, the second dose being given on the day next but one. The animals were killed 48, 48, 24 hr. and 5 days, resp., after the first injection, as indicated in parentheses in the Table. The incubation was carried out at pH 7.8 in a medium described in the text.

The mean values from 4 experiments are given expressed as μmoles citrate per mg. of nitrogen content in the sample, ± S.D.

Homogenate	Incubation time (min.)	Control (non-treated)	Group I (48 hr.)	Group II (48 hr.)	Group III (24 hr.)	Group IV (5 days)
Kidney	2	0.26 ± 0.03	0.31 ± 0.05	0.27 ± 0.02	0.25 ± 0.07	0.31 ± 0.03
	5	0.39 ± 0.05	0.48 ± 0.04	0.47 ± 0.05	0.38 ± 0.03	0.41 ± 0.01
	10	0.50 ± 0.07	0.67 ± 0.13	0.60 ± 0.12	0.45 ± 0.03	0.45 ± 0.07
	15	0.51 ± 0.07	0.64 ± 0.08	0.60 ± 0.10	0.47 ± 0.04	0.47 ± 0.11
	25	0.56 ± 0.09	0.69 ± 0.12	0.65 ± 0.13	0.45 ± 0.02	0.48 ± 0.10
Liver	5	0.60 ± 0.08	0.64 ± 0.03	0.62 ± 0.10	0.54 ± 0.07	0.53 ± 0.03
	10	0.90 ± 0.16	1.02 ± 0.16	0.99 ± 0.15	0.83 ± 0.13	0.86 ± 0.07
	15	1.31 ± 0.24	1.46 ± 0.07	1.36 ± 0.06	1.23 ± 0.24	1.12 ± 0.11
	25	1.63 ± 0.31	2.08 ± 0.32	2.17 ± 0.25	1.58 ± 0.36	1.62 ± 0.02

The fourth experiment was performed on four litters of five rats each; from every litter, one animal served as control; the other four were treated, respectively, with: both oestradiol benzoate and nicotinamide; nicotinamide only; oestradiol benzoate only; two doses of oestradiol benzoate. The kidney and liver homogenates were incubated at pH 7.8 (Table 4). An increase in the rate of citrate synthesis was observed in liver homogenates of rats treated with nicotinamide ($0.05 > P > 0.02$); this increase seems to be independent of the oestradiol benzoate treatment, as even a double dosis of oestradiol benzoate did not increase the ability of liver homogenates to synthetize citrate at the time when the excretion of citrate *in vivo* is diminished. The effect of nicotinamide on the kidney homogenates seems to be less pronounced.

Table 5

Chloride and α-ketoacids concentration in the blood plasma of female rats treated with oestradiol benzoate and nicotinamide

Blood plasma from animals used for the experiment described in Table 2 was taken for the determinations.
Chloride concentration is expressed as mE/l, α-ketoacids as μM/l ± S.D.

Compound	Control (non-treated)	Group I (13 hr.)	Group II (36 gr.)	Group III (60 hr.)
α-Ketoacids	410 ± 22	300 ± 27	320 ± 46	310 ± 57
Chloride	101 ± 3	107 ± 4	103 ± 4	105 ± 4

In Table 5, plasma α-ketoacids and chloride levels in the control rats and in rats subjected to the coupled oestradiol benzoate - nicotinamide treatment are presented. The α-ketoacids level was lower in all treated rats, whereas the plasma chloride level did not change.

DISCUSSION

The ability of the kidney and liver homogenates to synthetize citrate was increased when the rats had been treated previously with oestradiol benzoate and nicotinamide. The increased rate of citrate synthetis was independent of the time interval between the injection and the removal of tissue in the range from 13 to 60 hr. Even the kidney and liver homogenates of rats which had been killed at the time of decreased citrate excretion *in vivo* [7] did show an increased rate of citrate synthesis. The synthesis of citrate in the tissue preparations from animals treated with nicotinamide only did not differ from those treated with oestradiol benzoate coupled with nicotinamide. These results suggest that the observed increase of citrate synthesis in rat kidney and liver is caused by the intraperitoneal injection of nicotinamide.

Kaplan *et al.* [4] have shown that in several tissues the level of pyridine nucleotides is increased after an intraperitoneal injection of nicotinamide. Also the results of our experiments are consistent with the increased amount of pyridine nucleotides in the kidney and liver; NAD, taking part in the oxidative decarboxylation of pyruvate, may increase the amount of active acetate which is a substrate for the citrate condensing enzyme. As the equilibrium in the condensation reaction is shifted towards the synthesis of citrate the decarboxylation of pyruvate could be the limiting reaction and the concentration of the acetylo-CoA, the "pace-maker" of citrate synthesis. This view is supported by the observations that the differences in the rate of citrate synthesis were revealed only after a longer incubation and that the highest rate of synthesis was achieved at pH optimal for the oxidative decarboxylation of pyruvate. This assumption is supported also by the experiments of Marinello & Pallini [5] in which the rats had been injected intraperitoneally with nicotinamide, and the oxygen consumption by liver homogenates was measured in the presence of pyruvate; oxygen consumption following nicotinamide treatment was higher as compared with the control. The increased concentration of coenzyme A in rat liver observed by Mascitelli-Coriondoli [6] after nicotinamide injection seems also to support the possibility that the synthesis of active acetate is increased and hence the biosynthesis of citrate may go faster.

In the blood plasma of rats treated with oestradiol benzoate and nicotinamide a decrease in α-ketoacids concentration was found at the time of increased as well as decreased urine excretion of these acids. It may be supposed that also this effect results from the increased decarboxylation of α-ketoacids in the tissues. Plasma chloride concentration did not change significantly following oestradiol benzoate and nicotinamide treatment whereas the excretion of chloride in the urine was decreased.

SUMMARY

In connection with the previous finding that oestradiol benzoate and nicotinamide did change urine citrate excretion in female rats, citrate synthesis in rat tissues was studied. The rate of citrate synthesis from pyruvate and oxaloacetate catalysed by rat kidney and liver homogenates was increased when the rats had been treated either with oestradiol benzoate and nicotinamide or with nicotinamide alone. The treatment with oestradiol benzoate, which alone had no effect on the synthesis, enhanced the urine citrate excretion. In blood plasma after the incjection of oestradiol benzoate and nicotinamide, α-ketoacids decreased whereas the chloride level remained unchanged.

REFERENCES

[1] Beutler E., Yeh M. K. Y. & Ill B. A. - *J. Lab. Clin. Med.* **54**, 125, 1959.
[2] Friedmann T. E. & Haugen G. E. - *J. Biol. Chem.* **147**, 415, 1943.
[3] Homolka J., *Diagnostyka Biochemiczna* p. 312, Państwowy Zakład Wydawnictw Lekarskich, Warszawa 1958.
[4] Kaplan N. O., Goldin A., Humphreys S. R., Ciotti M. M. & Stoltzenbach F. E. - *J. Biol. Chem.* **219**, 287, 1956.
[5] Marinello E., Pallini V. - *R. esp. Fisiol.* **16**, suppl. II., 287, 1960.
[6] Mascitelli-Coriondoli E. - *Naturwiss.* **46**, 561, 1959.
[7] Żelewski L., Aleksandrowicz Z. & Dziadul C. - *Acta Biochim. Polon.* **9**, 351, 1962.
[8] Żelewski L. & Umiastowski J. - *Acta Biochim. Polon.* **9**, 153, 1962.
[9] Żelewski L., Żydowo M. & Purzycka J. - *Acta Biochim. Polon.* **9**, 147, 1962.

WPŁYW AMIDU KWASU NIKOTYNOWEGO I BENZOESANU ESTRADIOLU NA SYNTEZĘ CYTRYNIANU W NERCE I WĄTROBIE SZCZURA

Streszczenie

W związku z zaobserwowanymi u szczurów płci żeńskiej zmianami w wydalaniu kwasu cytrynowego po injekcji benzoesanu estradiolu i amidu kwasu nikotynowego podjęto badania nad syntezą kwasu cytrynowego w tkankach szczura. Stwierdzono wzrost syntezy kwasu cytrynowego z kwasu pirogronowego i szczawiooctowego w homogenatach nerkowych i wątrobowych szczurów po injekcji benzoesanu estradiolu i amidu kwasu nikotynowego. Wzrostu syntezy kwasu cytrynowego nie obserwowano po wyłącznym podaniu benzoesanu estradiolu. W osoczu po injekcji benzoesanu estradiolu i amidu kwasu nikotynowego stężenie α-ketokwasów ulega obniżeniu, podczas gdy stężenie chlorków pozostaje bez zmian.

Received 10 July 1963.

ACTA BIOCHIMICA POLONICA

Vol. XI 1964 No. 1

ALINA TAYLOR

PURIFICATION AND PROPERTIES OF THE Vi-PHAGE RECEPTOR FROM *SALMONELLA TYPHI* *

The Biochemical Laboratory, Institute of Marine Medicine, Gdańsk

The Vi-antigen is a substance produced by several of Enterobacteriaceae [3, 24,15] and composed mainly, if not entirely, of acetylated polyaminogalacturonic acid [11, 19]. The optical rotation of the Vi-antigen and its resistance to periodate oxidation suggest the α-1:3-glycoside linkages [5]. It is difficult to study the composition of the Vi-antigen owing to its unusual resistance to acid hydrolysis [1, 29, 5]. This resistance is explained by the influence of the carboxyl group and the protecting effect of the electropositive ammonium group, hindering the approach of H^+ ions to the glycosidic linkage [25].

It is generally assumed, although this has not been sufficiently confirmed, that the Vi-antigen is simultaneously a receptor for Vi-phages [7, 3, 21]. It is possible that the Vi-receptor constitutes only a part of the Vi-antigenic material of the bacterial cell. The purification of the Vi-substance is usually checked by serological tests. A technique for estimating the Vi-receptor activity has recently been developed in our Laboratory [28]. Simultaneous application of both methods facilitates the control of the course of purification.

The purification method developed for the Vi-antigen by Webster *et al.* [29, 31] proved unsuitable for Vi-receptor purification as the heating of the preparation with acetic acid caused a drastic drop in receptor activity. Baker *et al.* [2] applied the precipitation with cetyltrimethylammonium bromide (cetavlon). This method was not adopted owing to difficulties in removing cetavlon from the complex. The electrophoretic method reported by Jarvis *et al.* [12] could not be applied in our laboratory due to a lack of technical facilities. Koziński *et al.* [17] applied the formolized erythrocytes as selective adsorbent for the Vi-antigen. This method was adopted, with modifications consisting in the use of erythrocyte stroma, and the application of column chromatography.

* Partly supported by a grant from the Ministry of Health.

MATERIALS

Salmonella typhi 21802, Vi-phage type A, was obtained from the National Reference Laboratory for Enteric Phage Typing, Gdańsk. The bacteria were kept on egg medium and replated once a year after being checked by standard methods for serological and fermentative properties.

Vi-phage II type A was obtained from the International Reference Laboratory for Enteric Phage Typing, London.

Rabbit antisera against *Paracolobactrum ballerup* (serum anti-Vi ball), *Salmonella typhi* 21802 (serum anti-Vi, O ty) and *Salmonella typhi* O-58 (serum anti-O ty) were prepared by the National Reference Laboratory for Enteric Phage Typing, Gdańsk, and preserved with 0.2% of phenol.

Reagents: Dowex 50W × 8, Celite 535, and ninhydrin (B.D.H. England); pancreatine (K & K Laboratories, U.S.A., lot 18896 F); crystalline bovine serum albumin (Armour, U.S.A.), a gift from Dr. S. Angielski, tubings for dialysis (Kalle, West-Germany); Sephadex G25 (Pharmacia, Sweden). Galactosamine hydrochloride was isolated by Mr. R. Florczak from bovine trachea in this laboratory. Aminogalacturonic (2-amino-2-deoxy-D-galacturonic) acid hydrochloride was prepared from galactosamine hydrochloride according to the method of Heyns *et al.* [11]. The product was compared chromatographically with a sample kindly given by Prof. K. Heyns from the Hamburg University. Other reagents were of Polish origin.

METHODS

Vi-receptor activity: was estimated by the method previously described [28].

Precipitin test: was carried out after Landy & Webster [18]. The protein in the precipitates was determined according to Lowry *et al.* [20], crystalline bovine serum albumin being used as standard. The anti-Vi ball serum employed in this test was previously incubated at 37° with Vi-negative variant of *Salmonella typhi* 21802 till no agglutination of bacteria did occur. The amount of antigen which precipitates 100 μg. of antibody protein was taken as the antigen unit.

Haemagglutinin test: cock citrate blood was stored in the refrigerator not longer than a week. The procedure was carried out by setting up 0.1 ml. of two-fold serial dilutions of Vi-antigen, starting from the 0.1% solution, with 0.1 ml. portions of 1% suspension of washed cock erythrocytes. The dilutions were made on a plexiglass plate, using an automatic pipette.

After 1 hr. incubation at 37°, 0.2 ml. of the anti-Vi ball serum diluted 1:150 was added and the mixture incubated again at 37° for 1 hr. The quantity of haemagglutinin units per mg. of the preparation was read as the reciprocal of the end point dilution of the antigen (++ or +++). A solution containing 8.5 g. NaCl, 1.76 g. $Na_2HPO_4 \cdot 12H_2O$ and 0.1 g. KH_2PO_4 in 1 liter of water (pH 7.2) was used for all dilutions.

Electrophoresis: Immunoelectrophoresis was carried out on slides (25 × 75 mm.) covered with 4 ml. of 2% agar (Bacto-Difco) prepared in 0.1 M-diethylbarbiturate buffer, pH 8.6, or in 0.1 M-acetate buffer, pH 4.7. A voltage of 100 V was applied for 30 min., then the agar plates were incubated with antiserum in a moist box at room temperature for 2 or 3 days, and photographed.

Free electrophoresis was carried out in a Kern LK 30 apparatus using the same buffers as above. A voltage of 60 V was applied for 20 min.

Preparative electrophoresis was carried out in agar-gel (80 × 30 × × 8 mm.) at 90 V during 3.5 hr., the Bacto-Difco agar 1.5% in 0.1 M--diethylbarbiturate buffer, pH 8.6, being used. The agar was cut into 1 cm. strips and the fractions were detected by immunoelectrophoresis and eluted with water. The eluates were desalted on Sephadex G 25 column and lyophilized. The agar eluate served as control.

Physical properties. Specific viscosity ($\eta/\eta_0 - 1$) was measured at 20° in a capillary viscosimeter using 0.1% solution in 0.1 M-acetate buffer, pH 4.7. Optical rotation was examined in the Hilger-Watt polarimeter. Ultraviolet absorption spectrum was examined in Unicam SP-500 spectrophotometer, in 1 cm. cell, 0.25% solution in water being used. Infrared absorption spectrum was examined in a Perkin-Infracord spectrophotometer. The discs were pressed from 0.8 mg. of the Vi-receptor and 300 mg. of KBr.

Chemical analysis. Acetyl groups were determined according to Pregl & Roth [23] after 5 hr. hydrolysis in p-toluenesulphonic acid on a boiling water bath. For the estimation, acetanilide was used as standard. Phosphorus was determined according to Berenblum & Chain [4] and nitrogen according to Pregl & Roth [23]. Acid polysaccharides were determined by the turbidimetric method of Webster *et al.* [30]. The neutral equivalent was estimated by potentiometric titration to pH 7, after the solution of the Vi-receptor had been passed through Dowex 50 W X 8 H^+ column. Sugars were estimated by the anthrone method after Goebel & Barry [9] with a slight modification: before the addition of sulphuric acid, the solution was cooled on ice; this was found to improve the accuracy of the results.

RESULTS

Purification of the Vi-receptor

Preparation of the acetone-dried bacteria. The modified Stokes & Bayne culture medium [27, 28] was inoculated with *Salmonella typhi* and incubated at 38° till the concentration of bacteria, as measured by turbidity, amounted to about 10^9 cells/ml. (3 - 4 hr.). The suspension obtained was plated on the agar medium [16] containing 1% of peptone (Mikrokolor, Poland) and 0.1% sodium thiosulphate, in Petri dishes 20 cm. in diameter. After incubation at 38° for 18 - 20 hr. the bacteria were quickly washed off from the agar surface with 15 ml. water. The thick suspension was immediately poured into 3 vol. of acetone. After 1 hr. the supernatant was decanted and the bacteria were centrifuged at 2200 *g* for 3 min. The sediment was suspended in acetone and left overnight at 38° in order to kill the bacteria. During the next 3 days acetone was changed daily, and then the bacterial material was air-dried.

Preparation of crude Vi-receptor. The 2% suspension of dry bacteria collected from several seeds, in 0.9% NaCl solution was shaken with a few glass beads at room temperature exactly for 1 hr., then centrifuged for 20 min. at 14 000 *g*. To the clear, yellow supernatant 3 vol. of acetone were added. The precipitate was centrifuged for 3 min. at 2200 *g*, washed several times with acetone and dried. The resulting grayish powder, called *AP*, served as starting material for purification. As regards haemagglutination and receptor activities, the properties of this product were reasonably stable.

Acetone precipitation at pH 5 and pancreatine digestion. AP, 8.5 g., was suspended in 850 ml. of 0.01 N-acetic acid and was shaken for 1 hr. Then the yellow, turbid solution of pH 6 - 7 was adjusted to pH 5 with 1 N-acetic acid, mixed with 850 ml. of acetone, left for 1 hr. in the refrigerator and centrifuged at 2200 *g* for 15 min. The inactive supernatant was discarded and the sediment suspended in 500 ml. of phosphate-buffered saline (PBS) containing in 100 ml.: 0.80 g. NaCl, 0.22 g. KCl, 0.29 g. $Na_2HPO_4 \cdot 12H_2O$, 0.02 g. KH_2PO_4, 0.01 g. $CaCl_2$, and 0.01 g $MgCl_2 \cdot 6H_2O$. By this procedure a two-fold purification was obtained with no loss in activity.

Pancreatine, 290 mg., was suspended in 70 ml. of PBS, left for 15 min. at 37°, and the insoluble removed by centrifugation. The pancreatine solution was added to the suspension of the receptor preparation and the mixture dialysed against 3 - 4 lit. of PBS at 39° in a rotating bag, under toluene, PBS being changed every hour. After 4 hr. no protein was detectable by trichloroacetic acid and the mixture was dialysed overnight against running tap water, filtered to remove

the toluene, adjusted to pH 3 with acetic acid and added with 3 vol. of ethanol, and with sodium acetate to 0.1% concentration. The mixture was kept for 1 hr. in the refrigerator, then the sediment was collected by centrifuging at 2200 *g* for 5 min., dissolved in about 100 ml. of 0.1 M-NaCl and neutralized with a solution of sodium bicarbonate. The opalescent yellowish fluid was centrifuged for 30 min. at 18 000 *g*, the inactive sediment was discarded, and the clear fluid brought to a volume of 140 ml. with 0.1 M-NaCl. This preparation was denoted as *D* and was further purified by column chromatography.

Chromatography on erythrocyte stroma

Preparation of erythrocyte stroma. Human erythrocytes from 800 ml. of blood (from the Blood Donor Centre, Gdańsk) thoroughly washed with 0.15 M-NaCl were mixed with at least ten-fold volume of distilled water. After the sediment had settled, the supernatant was siphoned off and another portion of water was added. Washing was continued until the supernatant became colourless. The sediment was homogenized in the Pragomix homogenizer for 60 seconds, treated with distilled water and allowed to stand overnight at 0°. Then the stroma was centrifuged for 15 min. at 2200 *g* and washed with water until the supernatant became colourless. The sediment was suspended in 0.05 M-NaCl by mechanical stirring, saturated with carbon dioxide and the stroma was centrifuged as above. If this procedure had to be interrupted, it was necessary to wash the stroma with water and store it in the refrigerator. Washing was continued until the supernatant became colourless, then the stroma was suspended in 400 ml. of distilled water. The suspension was slowly added with 2 ml. of neutral 40% formaldehyde solution, with constant stirring, and stored in the refrigerator, sometimes even for several months. As for the preparation of the column for chromatography on erythrocyte stroma a known amount of stroma must be used, 5 ml. of the preparation was centrifuged before use in a graduated tube for 10 min. at 600 *g*, and from the volume of the sedimented stroma (approx. 0.3 - 0.4 ml.) the required volume of the stroma suspension was calculated.

Preparation of the column for chromatography. To the column (5 × × 12 cm.) provided with a Schott G-2 filter a suspension of Celite 535 in 0.2% formaldehyde solution was poured to form a layer 1 cm. thick. The mixture of 80 g. of Celite with the stroma suspension containing 40 ml. of stroma was poured into the column in 20 ml. portions. Before the next portion of the mixture was poured in, the upper part of the previously formed layer was cautiously stirred to prevent the formation of thin layers of stroma which would affect the flow. The column was washed successively with: 1 liter of 0.2% formaldehyde, 400 ml. of 0.5 M-NaCl, 400 ml. of water, 400 ml. of 0.5 M-NaCl, 400 ml. of water,

and finally with 1 liter of 0.2% formaldehyde; on the next day it was washed with 400 ml. of water, 400 ml. of 0.1 M-NaCl, 400 ml. of water and finally 200 ml. of 0.1 M-NaCl. A column thus prepared may be used for about 3 weeks and perform 12 - 15 adsorption-elution cycles, provided that it is washed with 0.2% formaldehyde after each cycle. If the work has to be discontinued for longer intervals, the column should be filled with 0.2% formaldehyde.

Chromatography. Half of the solution of the partially purified preparation *D* (70 ml.) was introduced slowly during 1 hr. into the column. The column was washed with 70 ml. of 0.1 M-NaCl and left overnight to permit the adsorption of the Vi-receptor. Then the column

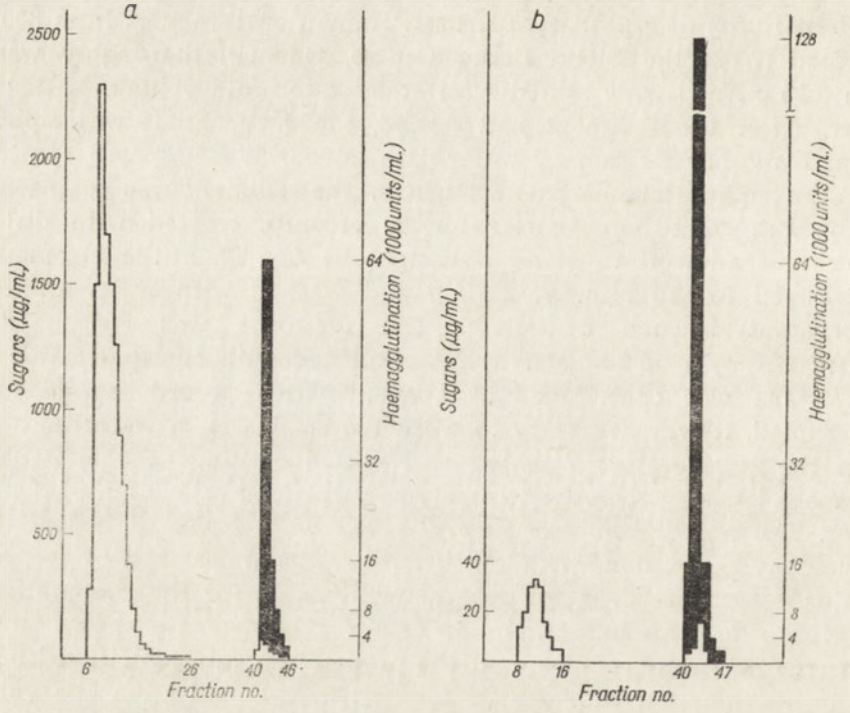


Fig. 1. Chromatography (*a*) and rechromatography (b) of the Vi-receptor preparation *D* on the erythrocyte stroma - Celite 535 column. The blackened space represents the haemagglutination, the unblackened one showing the content of sugars, estimated by the anthrone method. The elution was carried out by 0.1 M-NaCl and, starting from the 30th fraction, with water.

was eluted with 0.1 M-NaCl; 15 ml. fractions of the effluent were collected and tested for sugar by the anthrone method and for Vi-substance activity by haemagglutination (Fig. 1). The elution was continued until the effluent contained no sugar; usually the 30th fraction was sugar-free. Then the column was eluted with bidistilled water, the flow rate being adjusted to 15 ml. per 6 min. The appearance of the Vi-receptor in the effluent was manifested by foaming.

The fractions containing the Vi-receptor (usually 5 - 7 fractions) were pooled, dialysed overnight against distilled water, concentrated *in vacuo* to 25 ml. and centrifuged for 30 min. at 18 000 *g*. The supernatant fluid was almost clear and colourless, and this preparation of the purified Vi-receptor was denoted as *DC*.

Rechromatography. To 50 ml. of *DC* (obtained from two chromatographic runs) NaCl was added to 0.1M-concentration. The solution was introduced into the Celite-stroma column and chromatography was carried out as above (Fig. 1b). The active fractions were pooled, dialysed overnight against distilled water, concentrated *in vacuo* to a volume of 3 ml. and the impurities centrifuged off at 18 000 *g* for 30 min. This preparation of Vi-receptor was denoted as *DCR*.

Precipitation by ethyl ether. The ice-cooled *DCR* preparation (3 ml.) was mixed with 3 ml. of cooled 6M-formic acid, then with 18 ml. of cooled ethanol, left for 1 hr. in an ice bath and then centrifuged in a previously cooled tube for 5 min. at 600 *g*. The inactive sediment was discarded, to the supernatant 30 ml. of cooled ethyl ether was added, and the mixture left for 1 hr. in an ice-bath. The active precipitate was centrifuged as above and dissolved in 5 ml. of distilled water. The solution was dialysed against distilled water for 2 days, lyophilized, and the resulting purified Vi-receptor preparation was called *DCRE*.

The activity of the preparations in the course of three independent purification experiments is shown in Table 1. At successive steps of purification, samples were withdrawn, dialysed against distilled water, lyophilized, and used for activity estimations.

Vi-receptor purification with the omission of pancreatine. As one of the steps of purification consisted in pancreatine digestion, it was necessary to find out whether or not the receptor activity is affected by this treatment. Therefore the Vi-receptor preparation was purified with the omission of pancreatine digestion step. The *AP* preparation was treated with phenol at 65° according to Westphal *et al.* [34] and then purified by column chromatography as above. The activity of such Vi-preparation did not change on incubation with pancreatine [38].

Properties of the purified preparation

The purified Vi-receptor *DCRE* preparation was submitted to biological, chemical and physical examinations. Fig. 2 presents a typical curve of receptor activity estimation. The preparation showed high activity against Vi-phage II: 1 mg. of *DCRE* contained about 500 receptor units, one unit being defined as the ability to reduce the concentration of unadsorbed Vi-phage II by 10^{11}/ml. in the volume of 2 ml. Hence, under the conditions described, 1 mg. of the Vi-receptor preparation bonded 10^{14} ($500 \times 2 \times 10^{11}$) phage particles.

The purified Vi-receptor preparation had the properties of Vi-antigen. It evoked antibody formation (to be published), sensitized the erythrocytes to agglutination and was precipitated by anti-Vi serum. The precipitation curves for the purified *DCRE* preparation and the crude

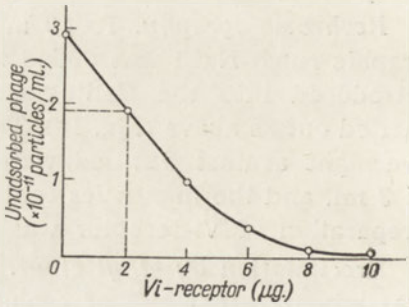


Fig. 2. Vi-receptor activity of the purified preparation (*DCRE*). In this preparation 2 μg. correspond to one receptor unit.

AP preparation are presented in Fig. 3; in both experiments the same anti-Vi ball serum was used. In the figure, the results of the examinations of the supernatants for excess of antigen or antibody are also included. The equivalence zone for *DCRE* was between 30 and 35 μg.

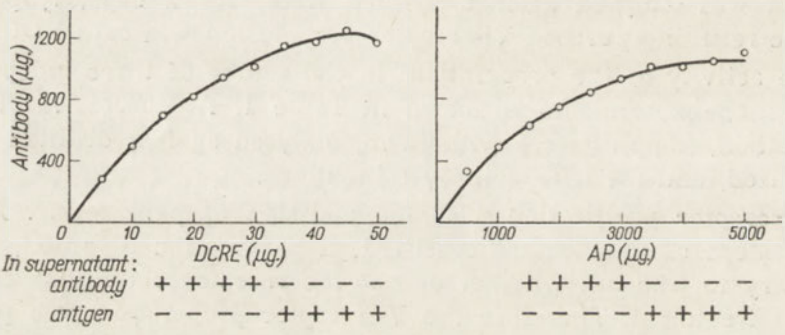


Fig. 3. Precipitation of the purified (*DCRE*) and crude (*AP*) Vi-receptor preparations by the anti-Vi ball serum. Below, the results of examinations of the supernatants for the presence of antibody or antigen are given.

that for *AP* between 3.0 and 3.5 mg. The purified Vi-receptor preparation tested by anti-O serum showed the absence of O-antigens. The haemagglutination by the Vi-receptor occurred already at a dilution of 1 mg./256 000 ml., so that 0.004 μg. sensitized 1 ml. of 1% cock erythrocyte suspension to agglutination by the anti-Vi serum.

Fig. 4 represents the immunoelectrophorograms (pH 8.6) of the Vi-receptor *DCRE* preparation and of the crude *AP* preparation. It is evident that the purified preparation did not contain any other antigens, present in the crude preparation. Two fractions of the Vi-antigen appeared in the purified preparation, antigenically identical [3] but differing in electrophoretic mobility. After electrophoretic separation

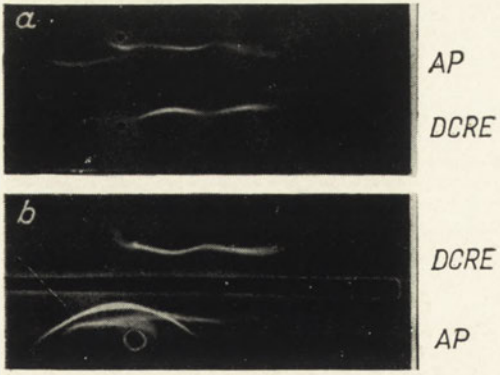


Fig. 4.

Fig. 5.

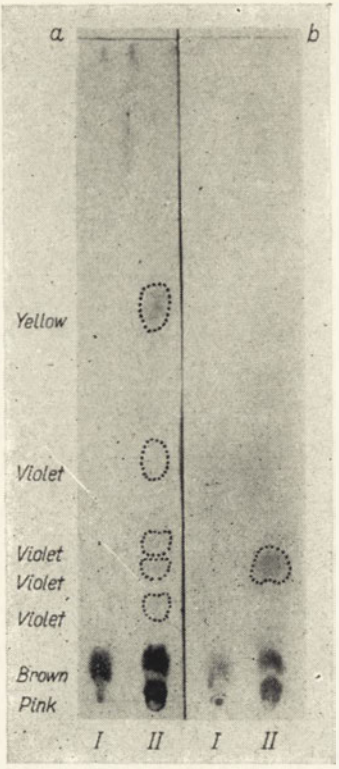


Fig. 7

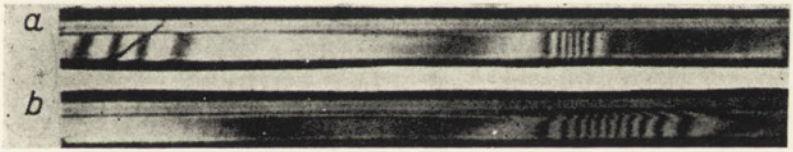


Fig. 6

Fig. 4. Immunoelectrophorograms of the purified (*DCRE*) and crude (*AP*) Vi-receptor preparations in agar-gel, pH 8.6, developed (*a*), with anti-Vi ball serum, and (b), with anti-Vi O ty serum.

Fig. 5. Immunoelectrophorogram of the purified Vi-receptor preparation (*DCRE*) in agar-gel, pH 4.7, developed with anti-Vi O ty serum.

Fig. 6. Free electrophoresis of the purified Vi-receptor preparation (*DCRE*) in (*a*), 0.1 M-diethylbarbiturate buffer, pH 8.6, and (b), 0.1 M-acetate buffer, pH 4.7.

Fig. 7. Chromatography of the hydrolysis products of the purified Vi-receptor preparation (conc. HCl, 2 hr., 100°) in phenol - water (4:1, v/v) + 0.1% of cupron. The spots were located (*a*), with ninhydrin, and (b), with aniline oxalate. *I*, Standard of aminogalacturonic acid: *II*, hydrolysis products of *DCRE*.

Table 1

Purification of the Vi-receptor

The results of 3 independent experiments, denoted by numbers 4, 5, and 6, are given. Details of purification are described in the text. The receptor activity of all preparations was estimated by the same Vi-phage II suspension. *AP* was digested by pancreatine before the Vi-receptor estimation [28].

Step of purification	Weight		Receptor activity (units)		Purification	Precipitin activity (units)		Purification	Haemaggl. activity (units/mg.)
	(mg.)	(%)	per mg.	total		per mg.	total		
AP	8 500	100	1.0	8 500		5.2	44 200		800
D-4	1 333	16	5.9	7 900	5.9	25.0	33 400	4.8	4 000
D-5	1 247	15	7.4	9 200	7.4	25.0	31 200	4.8	4 000
D-6	1 192	14	7.4	8 800	7.4	27.8	33 200	4.9	8 000
DC-4	46.2	0.54	200	9 200	200	400	18 500	77	128 000
DC-5	52.8	0.62	190	10 000	190	380	20 100	73	128 000
DC-6	58.3	0.68	210	12 200	210	380	22 200	73	128 000
DCR-4	24.4	0.29	420	10 200	420	530	12 900	102	128 000
DCR-5	31.2	0.35	330	10 300	330	440	13 700	85	256 000
DCR-6	32.4	0.38	310	10 000	310	480	15 500	92	128 000
DCRE-4	16.3	0.19	590	9 600	590	590	9 600	113	256 000
DCRE-5	19.9	0.23	500	10 000	500	470	9 400	90	256 000
DCRE-6	21.6	0.25	470	10 200	470	540	11 700	104	256 000

on agar-gel the slower moving fraction showed receptor and haemagglutination activities, whereas the faster moving fraction possessed neither of them. At pH 4.7 there appeared two fractions with the same mobility but producing separate precipitin bands with anti-Vi antibodies (Fig. 5).

The results of free electrophoresis are presented in Fig. 6. At pH 8.6 two groups of interference fringes appeared, indicating the presence of two fractions; at pH 4.7 there appeared only one group of interference fringes. These results are consistent with those obtained by immunoelectrophoresis. The infrared spectrum (Fig. 8) is very similar to the

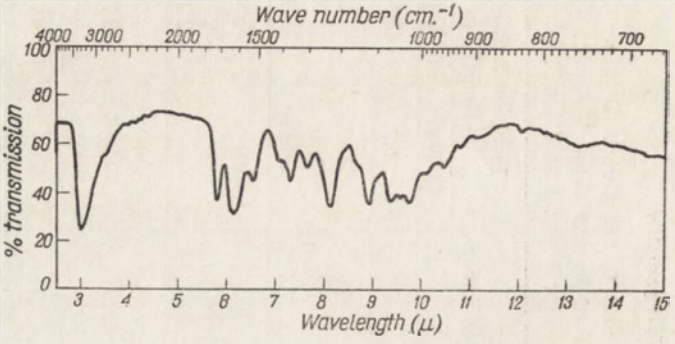


Fig. 8. Infrared spectrum of the purified Vi-receptor preparation (*DCRE*).

spectra presented by Webster *et al.* [31]. The ultraviolet spectrum (Fig. 9) has a slight peak at 260 mμ, with $E^{1\%}_{1\,cm}$ amounting to 1.5; this may indicate the presence of nucleotide contamination of about 0.5%, and is in agreement with the results of phosphorus estimation

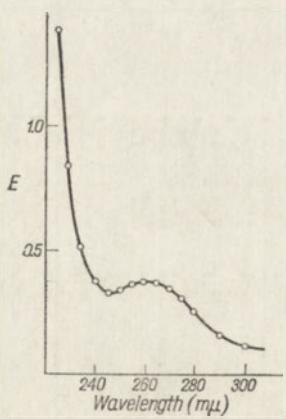


Fig. 9. Ultraviolet spectrum of the purified Vi-receptor preparation (*DCRE*). Conditions: 0.25% water solution, 1 cm. cell.

(0.1% P). The results of the analysis of the purified Vi-receptor preparation are summarized in Table 2.

The Vi-receptor preparation was subjected to hydrolysis under optimum conditions given by Clark *et al.* [5] and Webster *et al.* [32]

Table 2

Analysis of the purified Vi-receptor preparation (DCRE)

The calculations were made without considering the ash contents.

C (%)	40.7
H (%)	6.27
N (%)	5.39
P (%)	0.1
Acetyl groups (%)	19.3
Neutral equivalent	280
Acidic polysaccharides (turbid. units/mg.)	1910
Specific viscosity ($(\eta/\eta_0 - 1)$	0.24
$[\alpha]_D^{18}$	+280
Ash (%)	1.5

(conc. HCl, 2 hr., 100°). The hydrolysates were examined by the descending paper chromatography, 300 μg. of the hydrolysate being applied at a time. The following solvent systems were examined (all proportions are given by volume): (*a*), phenol - water (4:1, v/v), with 0.1% cupron; (*b*), *n*-butanol - acetic acid - water (38:12:50); (*c*), *n*-butanol - ethanol - water (40:10:50); (*d*), *n*-butanol - pyridine - water (30:20:15); (*e*), *n*-amyl alcohol - pyridine - water (14:14:12).

The best separation was obtained in the phenol - water system, the spots being located with ninhydrin and aniline oxalate (Fig. 7). Two main spots were found; one corresponded to aminogalacturonic acid, another to a substance, which seemed to be an unhydrolysed fragment, as after isolation and further hydrolysis it released aminogalacturonic acid. The hydrolysis with 2 N-HCl at 100° for 2 hr., and with 6 N-HCl at 105° for 25 hr. liberated only trace amounts of substances reacting with aniline oxalate or ninhydrin.

The two fractions separated by the agar-gel electrophoresis were eluted and hydrolysed with conc. HCl. The descending paper chromatography in the solvent systems *a* and *b* was performed. The chromatograms were developed with ninhydrin or aniline oxalate, and no differences between the two fractions were found in the position, colour or intensity of the spots.

DISCUSSION

The described method permits to obtain by means of a very mild procedure a Vi-phage receptor preparation of considerable degree of purity. The improvements adopted consist chiefly in the omission of reactions such as heating with acid, and of detergents such as quarternary ammonium salts which may cause depolymerization [22]. It should be emphasized that reproducible results were obtained (Table 1).

The Vi-receptor preparation was purified 520-fold in relation to the starting material, *AP*. As *AP* constituted 20 - 25% of bacterial dry weight, the degree of purification in relation to dry bacteria amounted to about 2000. Hence the content of the receptor substance in dry bacteria would be about 0.05%. From the estimation of Vi-receptor activity it follows that 1 μg. of the preparation binds 10^{11} phage particles; this value is of the same range as the activity of the T5 phage receptor preparations of Weidel *et al.* [33].

During the purification of Vi-receptor no loss of total activity did occur, on the contrary, even an increase in activity was observed. This may be attributed to some inhibitor(s) present in the starting material which is not removed by pancreatine digestion. However, one may assume that the inhibition is but slight, for in the previous experiments [28] full receptor activity was revealed after pancreatine digestion of the mixture of purified Vi-receptor and *AP* from the Vi-negative variant of *S. typhi*.

The results of quantitative precipitin tests made in the course of purification suggest that the Vi-receptor substance constitutes only a part of the Vi-antigenic material of *Salmonella typhi*. The purification degree, measured by the precipitin test, amounts to about 100 in relation to *AP*. As the yield of the precipitin activity amounts on the average only to 20%, this test cannot serve as a reliable measure of the Vi-receptor content in the material examined. On the other hand, the results of the haemagglutinin test roughly correspond with the results of the receptor estimation, and therefore haemagglutination can be applied as a quick method for approximate estimations.

The presented results permit to compare the properties of the Vi-receptor preparation with those of the Vi-antigen preparation from *Salmonella typhi*, obtained by other authors. The nitrogen contents, neutral equivalents, optical rotation values and infrared spectra are similar, but there are differences in the acetyl contents, viscosity, and turbidimetric estimations. The Vi-receptor preparation possesses more acetyl groups (19.3%), is more viscous (specific viscosity 0.24) and gives higher values in turbidimetric estimations of acid polysaccharides (1920 units/mg.) than the Vi-antigen of Webster *et al.* [31] (12% acetyl, specific viscosity 0.08, and 660 turbidimetric units/mg.). The Vi-antigen preparation of Baker *et al.* [2] possessed similar contents of acetyl groups (20.45 - 24.30%); the viscosity could not be compared as the authors did not describe the conditions of estimation. The observed differences in the Vi-receptor properties may be due to the mild conditions of purification applied, which probably did not cause deacetylation or depolymerization. On the other hand, however, these results may reflect the differences between the *Salmonella typhi* strains used.

The obtained Vi-receptor preparation was immunologically pure since neither by the precipitin test nor by immunoelectrophoresis any other antigen, beside the Vi-antigen, was detected. No sugars characteristic of O-antigens of *Salmonella typhi* [26] were found by chromatographic analysis of the hydrolysates. Beside intensive spots of the aminogalacturonic acid and the unhydrolysed polymer fragment, on the chromatogram 3 - 4 slight ninhydrin-positive spots were present. They either correspond to a component(s) forming a very small proportion of the Vi-receptor, or to some impurities. The contamination with nucleic acids or their degradation products did not exceed 0.5% as shown by UV spectra and by phosphorus determination (0.1%).

The immunoelectrophoresis of the Vi-receptor preparation revealed the presence of two fractions, differing in electrophoretic mobility. Both these fractions were also present in the original material. Both were precipitated by anti-Vi antibodies in the same manner, revealing antigenic identity, but they differed in biological activity as only the slower moving fraction was active in the receptor and heamagglutinin tests. As no differences in their composition by the chromatography of their hydrolysates could be found, it may be suggested that the differences in their biological properties are connected with differences in acetyl groups content or in degree of polymerization.

Polydispersion of the Vi-antigen has been repeatedly reported. By the gel diffusion method, Whiteside & Baker [35] found two fractions which differed in the acetyl group content [36]. Landy *et al.* [19] demonstrated that the deacetylation of the Vi-antigen reduced markedly its serological activity, which could be restored to a great extent by reacetylation. Jefimow [13] observed fractions of different mobility in immunoelectrophoresis, of preparations obtained by the method of Webster *et al.* (slightly modified). Using the same preparations, Kamienskaja [14] demonstrated the presence of several Vi-antigenic components differing in the serological cross-reactions. It is possible that the bacterial cell produces a series of aminogalacturonic acid polymers differing in antigenic or haptenic properties. This view seems to support the assumption that the Vi-receptor represents only a part of the Vi-antigenic material of the bacterial cell.

I wish to thank Prof. Dr. E. Mikulaszek for guidance of this study and Prof. Dr. Z. Buczowski for his interest throughout the experiments and for supplying me with sera and bacteria strains. Thanks are due to Doc. Dr. J. Bartz for the infrared estimations and to Prof. Dr. D. Shugar, Doc. Dr. T. Jasiński and Dr. J. Sokołowski for the use of apparatus for physical and chemical studies. I am also indebted to Mrs. J. Żabina and Mrs. J. Starczewska for their valuable technical assistance.

SUMMARY

The purification of Vi-phage receptor from *Salmonella typhi* 21802 is described. The procedure consists in pancreatine digestion, chromatography on human erythrocyte stroma set on Celite, and precipitation with ether from the water - ethanol - formic acid solution. The degree of purification measured by receptor activity in relation to dry bacterial wt. amounts to 2000. The preparation possesses Vi-antigenic properties. The chromatograms of the Vi-receptor hydrolysates indicate the presence of aminogalacturonic acid. The immunoelectrophoresis of the Vi-receptor preparation reveals the presence of two fractions, showing antigenic identity. Only the slower one is active in the receptor and haemagglutinin tests. Probably Vi-receptor represents only a part of the Vi-antigenic material of the bacterial cell.

REFERENCES

[1] Ashida T. - *Japan J. Exper. Med.* **20**, 181, 1949.
[2] Baker E. E., Whiteside R. E., Basch R. & Derow M. A. - *J. Immunol.* **83**, 680, 1959.
[3] Baron L. S., Formal S. B. & Spilman W. - *J. Bact.* **69**, 177, 1955.
[4] Berenblum J. & Chain E. - *Biochem. J.* **32**, 259, 1938.
[5] Clark W. R., McLaughlin J. & Webster M. E. - *J. Biol. Chem.* **230**, 81, 1958.
[6] Crowle A. J., *Immunodiffusion.* Acad. Press. New York 1961.
[7] Edlinger E. & Vieuchange J. - *Ann. Inst. Pasteur* **84**, 368, 1953.
[8] Felix A. & Pitt R. M. - *Lancet* **227**, 186, 1934.
[9] Goebel W. F. & Barry G. T. - *J. Exptl. Med.* **107**, 185, 1958.
[10] Heyns K. & Paulsen H. - *Chem. Ber.* **88**, 188, 1955.
[11] Heyns K., Kiessling G., Lindberg W., Paulsen H. & Webster M. E. - *Chem. Ber.* **92**, 2435, 1959.
[12] Jarvis F. G., Mesenko M. T. & Kyle J. E. - *J. Bact.* **80**, 677, 1960.
[13] Jefimow D. D. - *Trudy Moskowskogo Nauczno-Issliedowatielskogo Instituta Epidiemiołogii i Mikrobiołogii Ministierstwa Zdrawochranienija RSFSR.* **9**, 76, 1962.
[14] Kamienskaja I. N. - *Trudy Moskowskogo Nauczno-Issliedowatielskogo Instituta Epidemiołogii i Mikrobiołogii Ministierstwa Zdrawochranienija RSFSR.* **9**, 86, 1962.
[15] Kauffmann F. & Møller E. - *J. Hyg.* **40**, 248, 1940.
[16] Kauffmann F., *Enterobacteriaceae*, p. 354. Ejnar Munksgaard Publisher. Copenhagen 1954.
[17] Koziński A. W., *Biologia wirusów* (E. Mikulaszek & W. Dobrzański, eds.) p. 41. PWN. Warszawa 1956.
[18] Landy M. & Webster M. E. - *J. Immunol.* **69**, 143, 1952.
[19] Landy M., Johnson A. G. & Webster M. E. - *Am. J. Hyg.* **73**, 55, 1961.
[20] Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. - *J. Biol. Chem.* **193**, 265, 1951.
[21] Mikulaszek E. - *Ann. Inst. Pasteur* **91** (suppl), 40, 1956.
[22] Morgan W. T. J., in *Chemistry and Biology of Mucopolysaccharides* (G. E. W. Wolstenholme & M. O'Connor, eds.) p. 18. J. A. Churchill. London 1958.

[23] Pregl F. & Roth H., *Quantitative organische Mikroanalyse*. Springer Verlag. Vien 1958.
[24] Rouchdi M. - *Compt. Rend. Soc. Biol.* **128**, 1022, 1938.
[25] Stacey M. in *Chemistry and Biology of Mucopolysaccharides* (G. E. W. Wolstenholme & M. O'Connor, eds.) p. 6. J. A. Churchill. London 1958.
[26] Staub A. M., Tinelli R., Lüderitz O. & Westphal O. - *Ann. Inst. Pasteur* **96**, 303, 1959.
[27] Stokes J. L. & Bayne H. G. - *J. Bact.* **76**, 417, 1958.
[28] Taylor K. & Taylor A. - *Acta Microbiol. Polon.* **12**, 97, 1963.
[29] Webster M. E., Landy M. & Freeman M. E. - *J. Immunol* **69**, 135, 1952.
[30] Webster M. E., Sagin J. F. & Freeman M. E. - *Proc. Soc. Biol. Med.* **81**, 263, 1952.
[31] Webster M. E., Sagin J. F., Anderson P. R., Breese S. S., Freeman M. E. & Landy M. - *J. Immunol.* **73**, 16, 1954.
[32] Webster M. E., Clark W. R. & Freeman M. E. - *Arch. Biochem. Biophys.* **50**, 223, 1954.
[33] Weidel W., Koch G. & Bobosch H. - *Z. Naturforsch.* **9B**, 573, 1954.
[34] Westphal O., Lüderitz O. & Bister F. - *Z. Naturforsch.* **7B**, 148, 1952.
[35] Whiteside R. E. & Baker E. E. - *J. Immunol.* **83**, 687, 1959.
[36] Whiteside R. E. & Baker E. E. - *J. Immunol.* **84**, 221, 1960.

OTRZYMYWANIE I WŁASNOŚCI OCZYSZCZONEGO PREPARATU RECEPTORA Vi Z BAKTERII *SALMONELLA TYPHI*

Streszczenie

Opisano metodę oczyszczania receptora faga Vi z *Salmonella typhi* 21802. Metoda polega na trawieniu pankreatyną, chromatografii na błonach krwinek osadzonych na celicie i wytrącaniu eterem z roztworu zawierającego etanol, kwas mrówkowy i wodę. Stopień oczyszczenia w stosunku do suchej masy bakteryjnej wynosi 2000 (na podstawie oznaczeń aktywności receptorowej).

Preparat posiada własności antygenu Vi. Na chromatogramach hydrolizatów receptora Vi stwierdzono obecność prawie wyłącznie kwasu aminogalakturonowego. W immunoelektroforezie preparat rozdziela się na dwie frakcje wykazujące identyczność antygenową. Tylko wolniejsza frakcja posiada aktywność receptorową i daje reakcję hemaglutynacji. Prawdopodobnie receptor Vi stanowi tylko część materiału antygenowego Vi komórki bakteryjnej.

Received 12 July 1963

ACTA BIOCHIMICA POLONICA

Vol. XI 1964 No. 1

B. ZAGALAK and J. PAWEŁKIEWICZ

SYNTHESIS AND PROPERTIES OF SOME ANALOGUES OF THE CORRIN COENZYMES

Department of Biochemistry, College of Agriculture, Poznań

A special type of linkage, i.e. the organometallic bond between the atom of cobalt and the carbon atom $C_{(5)}$ of 5′-deoxyadenosyl group is present in the molecule of coenzyme B_{12} [13]. Recently, chemical synthesis of the coenzyme B_{12} has been described; it was based on the reaction of cobamide hydride with 5′-*O*-tosyl-2′,3′-*O*-isopropylidene adenosine and on subsequent removal of the isopropylidene protecting group from the obtained product [3, 16, 21]. With this method the synthesis of several analogues of corrin coenzymes could be attempted. In the analogues, the deoxyadenosyl can be replaced by other deoxynucleoside groups, or the hydrides of other corrin derivatives can be used in the place of cobamide hydride for the synthesis of other analogues. These studies may provide an opportunity to elucidate the relation between the structure and function of coenzyme B_{12}, and to synthetize an "anticoenzyme B_{12}" which could be used in metabolic studies and in chemotherapy.

In this paper the synthesis of several new corrin coenzymes and their properties are described. The biological activity of these analogues was tested in the coenzyme B_{12} dependent enzymic reaction, in which glycerol is converted into β-hydroxypropionaldehyde [22].

MATERIALS AND METHODS

Vitamin B_{12} (5,6-dimethylbenzimidazolylcobamide cyanide, DMBIA--cobamide cyanide) and aquocobinamide cyanide (Factor B) were isolated from cultures of *Propionibacterium shermanii* [22]. 3,5,6-Trimethylbenzimidazolylcobamide cyanide (TMBIA-cobamide cyanide) was obtained by methylation of vitamin B_{12} with dimethyl sulphate in the presence of KCN [7]. The crude product of the methylation after desalting by phenol extraction was purified by paper chromatography. The chromatograms were run on Whatman no. 3 paper in *n*-butanol - pro-

pan-2-ol - water - acetic acid (100:70:99:1, by vol.) in the atmosphere of HCN. The natural coenzyme B_{12} and cobinamide coenzyme were isolated from cultures of *P. shermanii* and the pseudovitamin B_{12} coenzyme (Co-5′-dAdo-*a*-adenylcobamide) from the culture of *P. arabinosum* [23]. The coenzyme form of *a,b,c,d,e,g*-hexamidecobyrinic acid (Co-5′-dAdo-*a,b,c,d,e,g*-hexamidecobyrinic acid) was isolated from cultures of *Nocardia rugosa* (mutant 466) [15]. Co-alkyl corrinoid derivatives were obtained according to the methods previously described [22]. 5′-Tosyl-2′,3′-*O*-isopropylidene nucleosides were obtained by the action of *p*-toluenesulfonyl chloride on the respective isopropylidene nucleoside derivatives in pyridine solution. The 2′,3′-*O*-isopropylidene derivatives of nucleosides were obtained in good yields according to the method of Hampton & Magrath [10]. This is based on the condensation of nucleoside with anhydrous acetone in the presence of an excess of *p*-toluenesulfonic acid. 2′,3′-Isopropylidene adenosine was obtained as described by Hampton [9], the uridine analogue according to Ikehara *et al.* [11], and the cytidine analogue according to Chambers *et al.* [5]. The purity of the obtained substances was checked by chromatography on Whatman no. 1 paper in propan-2-ol - water - ammonia (70:25:5, by vol.) [9]. The synthesis of 5′-*O*-tosyl-2′,3′-isopropylidene adenosine was performed according to Sakami & Stevens [19], the respective uridine and cytidine analogues being prepared according to Levene & Tipson [14] and Clark *et al.* [6]. As it was shown by paper chromatography on Whatman no. 1 paper in ethanol - ammonia - water (80:4:16, by vol.) [6] and in *n*-butanol - acetic acid - water (4:1:5, by vol.) [19], the products, except the uridine derivative, contained some small amount of unidentified impurities; nevertheless they were used for subsequent syntheses of corrinoid analogues without further purification.

The enzymic system converting glycerol into β-hydroxypropionaldehyde was prepared from the cells of *Aerobacter aerogenes* (no. 572) according to the method previously described [24, 22]. β-Hydroxypropionaldehyde was assayed by the method of Smiley & Sobolov [20]. Estimations of acetaldehyde or propionaldehyde formed in the enzymic reactions from ethylene glycol or propan-1,2-diol, respectively, were performed according to the method of Böhme & Winkler [4] with the 2,4-dinitrophenylhydrazine test. The Bausch & Lomb photocolorimeter Spectronic 20 was used.

Spectral analyses were performed on a Hilger H 700 spectrophotometer in silica cells (length 10 mm.). The concentrations of dicyanide corrinoids were estimated by measuring the extinctions at 580 mμ. For the calculations, the molar extinction coefficient of 10.1×10^3 [1] for all dicyanide corrinoids [18] was used.

For purification and identification of the obtained corrinoid analogues, paper chromatography and paper electrophoresis in dark room

were employed. The chromatograms were run by the descending technique on Whatman no. 3 paper in: (*A*), *n*-butanol - propan-2-ol - - water - acetic acid (10:70:99:1, by vol.) or (*B*), *n*-butan-2-ol saturated with water.

The electrophoretic separation was made on Whatman no. 3 paper in 1 N-acetic acid at the potential of 6 - 8 V/cm.

The nucleosides used in these studies were commercial products; adenosine and cytidine, Nutritional Biochemicals Corporation, U.S.A.; uridine, Reanal (Hungary). Other chemicals were from Fabryka Odczynników Chemicznych, Gliwice (Poland).

RESULTS AND DISCUSSION

Synthesis of analogues of the coenzymes

The syntheses were performed as follows: 20 mg. of corrinoid cyanide (DMBIA-cobamide cyanide, cobinamide cyanide or TMBIA-cobamide cyanide) was dissolved in 2 ml. of 10% aqueous solution of ammonium chloride, and 100 mg. of zinc fillings and 1 mg. of magnesium powder were added. The reduction was performed in the atmosphere of nitrogen, the sample being shaken for 5 - 10 min. until the grey-green hydride was obtained. Then, in the dark, to the solution 8 mg. of 5′-tosyl-2′,3′-isopropylidene nucleoside (the derivative of adenosine, cytidine or uridine) dissolved in 0.2 ml. of ethanol was added with vigorous shaking, care being taken to avoid oxygen. The mixture was left for 5 min., then 20 ml. of water was added and the mixture filtered. The solution was adjusted with acetic acid to pH 5 and the corrin compounds were extracted with a mixture of phenol and trichloroethylene (1:1, v/v). They were re-extracted from the phenolic solution with water after the addition of 10 volumes of acetone. From the water solution acetone was distilled off and the derivatives of the coenzyme analogues were hydrolysed with hydrochloric acid to remove the isopropylidene group. For this purpose 1 volume of 2 N-HCl was added and the sample left for 30 min. at 25 - 30°. The solution was then neutralized with ammonia and desalted with phenol as described above. The solution of corrinoids free of inorganic salts was evaporated at room temperature over solid KOH under reduced pressure. The crude product of the synthesis was purified, adenosyl and cytidyl analogues by electrophoresis in 1 N-acetic acid, and uridyl analogues by chromatography in the solvent system *A*. The band of the analogue was cut off, eluted, and the compound subjected to further purification either by paper chromatography (samples isolated by electrophoresis) or by paper electrophoresis (samples isolated by paper chromatography). The products thus purified were used for subsequent studies.

It was found that in the synthesis of the corrin coenzymes analogues the yields were improved in the absence of oxygen. In the presence of oxygen, from corrinoid hydrides were formed hydroxyls which do not react with tosyl derivatives of the nucleosides. When, under the conditions described, instead of corrin cyanides the hydroxyl derivatives were used, no differences in the yields of the analogues were observed. The highest yields were obtained with the uridine analogue; the only by-products observed were the unchanged corrinoid cyanide and its hydroxylic forms. On the other hand, in the course of synthesis of adenosyl and cytidyl analogues the by-products amounted to 20 - 30%. They exhibited weaker basic properties than the main product and on electrophoresis moved similarly as the Co-alkyl derivatives of corrinoids. These compounds contained the nucleoside group but their amino groups were probably substitued by tosyl residues. No detailed studies on these derivatives were made.

Physico-chemical properties of the analogues

The formulae of the synthetized coenzyme analogues are shown in Figs. 1 a and 1 b. Also the naturally occurring coenzyme B_{12} and cobinamide coenzyme were synthetized and the two synthetic products possessed all the properties of the compounds isolated from microorganisms.

The incorporation of nucleoside in chemical synthesis was confirmed by the isolation of the nucleoside after photolysis of Co-5′-dAdo-TMBIA-cobamide (formula VII). In this experiment about 2 mg. of the compound in aqueous solution was exposed to light until complete conversion into the hydroxylic form. The products were separated by chromatography on Whatman no. 1 paper in *n*-butanol - water - ammonia (40:50:5, by vol. [2]). The corrinoid remained on the start line while adenosine and its derivatives moved with the solvent. In ultraviolet light a spot with the mobility of adenosine was detected. The spot was cut off and eluted with 0.01 N-HCl. The absorption spectrum of the eluate exhibited a maximum at 258 mμ in 0.01 N-HCl, and at 262 mμ in 0.1 N-NaOH solution, indicating the presence of $N_{(9)}$-substituted derivative of adenine [8, 12].

The rates of movement of the corrin coenzyme analogues on the paper chromatogram relative to coenzyme B_{12} (Table 1) were, as it could be expected, similar. These values for the less hydrophilic Co-methyl derivatives of vitamin B_{12} and of cobinamide, included for comparison, differ largely from those for nucleoside analogues. The electrophoretic mobility of the compounds studied is shown in Table 1. As the differences in mobility are considerable, paper electrophoresis forms a convenient method for separation and purification of nucleoside analogues.

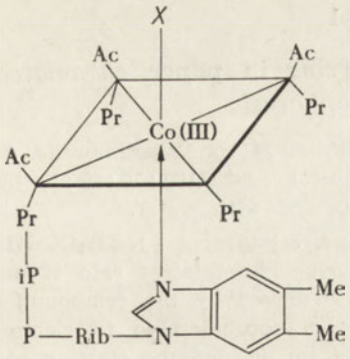


Fig. 1a

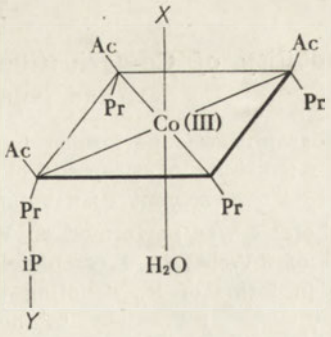


Fig. 1b

Fig. 1. Schemes of structural formulae of the synthetized analogues of corrin coenzymes, their systematic names and abbreviations used.
Ac, $-CH_2CONH_2$; Pr, $-CH_2CH_2CONH_2$; P, phosphoric acid; iP, isopropanolamine; Rib, ribofuranose.

Fig. 1a

I X = 5′-deoxyadenosyl
Co-5′-deoxyadenosyl-α-(5,6-dimethylbenzimidazolyl)-cobamide
Co-5′-dAdo-DMBIA-cobamide (coenzyme B_{12})

II X = 5′-deoxycytidyl
Co-5′-deoxycytidyl-α-(5,6-dimethylbenzimidazolyl)-cobamide
Co-5′-dCyd-DMBIA-cobamide

III X = 5′-deoxyuridyl
Co-5′-deoxyuridyl-α-(5,6-dimethylbenzimidazolyl)-cobamide
Co-5′-dUrd-DMBIA-cobamide

Fig. 1b

IV X = 5′-deoxyadenosyl Y = H
Co-5′-deoxyadenosyl-aquocobinamide
Co-5′-dAdo-cobinamide (cobinamide coenzyme)

V X = 5′-deoxycytidyl Y = H
Co-5′-deoxycytidyl-aquocobinamide
Co-5′-dCyd-cobinamide

VI X = 5′-deoxyuridyl Y = H
Co-5′-deoxyuridyl-aquocobinamide
Co-5′-dUrd-cobinamide

VII X = 5′-deoxyadenosyl Y = α-(3,5,6-trimethylbenzimidazolyl)-ribofuranosyde-3′-phosphate
Co-5′-deoxyadenosyl-α-(3,5,6-trimethylbenzimidazolyl)-aquocobamide
Co-5′-dAdo-TMBIA-cobamide

VIII X = 5′-deoxycytidyl Y, as in VII
Co-5′-deoxycytidyl-α-(3,5,6-trimethylbenzimidazolyl)-aquocobamide
Co-5′-dCyd-TMBIA-cobamide

IX X = 5′-deoxyuridyl Y, as in VII
Co-5′-deoxyuridyl-α-(3,5,6-trimethylbenzimidazolyl)-aquocobamide
Co-5′-dUrd-TMBIA-cobamide

Table 1

The mobility of Co-derivatives of corrins in paper chromatography and paper electrophoresis

Chromatography was performed in dark room at 24° on Whatman no. 3 paper. Solvent A: *n*-butanol - propan-2-ol - water - acetic acid (100 : 70 : 99 : 1, by vol.); solvent B: *n*-butan-2-ol saturated with water.

Electrophoresis was performed on Whatman no. 3 paper in 1 N-CH_3COOH at the potential of 8 V/cm., in a completely dark room. The relative rate of migration is given in terms of R_B denoting the distance moved by the compound divided by the distance moved by cobinamide. As cyanocobalamine is electroneutral at pH 2.7, its position on the electrophorogram defined the starting point.

No.	Compound	Paper chromatography		Paper electrophoresis
		Solv. A	Solv. B	
		$R_{\text{coenzyme } B_{12}}$		R_B
	Cyanocobalamine			0.00
	Aquocobinamide cyanide			1.00
I	Co-5′-dAdo-DMBIA-cobamide	1.00	1.00	1.18
IV	Co-5′-dAdo-cobinamide	1.08	1.14	1.60
VII	Co-5′-dAdo-TMBIA-cobamide	0.88	1.08	1.31
II	Co-5′-dCyd-DMBIA-cobamide	0.84	0.82	1.34
V	Co-5′-dCyd-cobinamide	0.92	0.80	1.74
VIII	Co-5′-dCyd-TMBIA-cobamide	0.86	0.80	1.47
III	Co-5′-dUrd-DMBIA-cobamide	0.87	0.87	0.64
VI	Co-5′-dUrd-cobinamide	1.06	1.15	0.98
IX	Co-5′-dUrd-TMBIA-cobamide	1.00	1.18	0.78
	Co-methyl-DMBIA-cobamide	1.62	2.22	0.50
	Co-methyl-cobinamide	1.62	2.00	0.98

Similarly as the natural compounds, all the synthetized analogues of corrin coenzymes are light-sensitive. In Table 2 are summarized the data on the rate of degradation of these compounds. The most sensitive ones are coenzyme B_{12} (formula I) and its cytidine (II) and uridine (III) analogues. The analogues of cobinamide (V and VI), on the other hand, are more stable even when compared with the natural cobinamide coenzyme (IV). In this respect they differ from the Co-alkyl derivatives of cobinamide, which on exposure to light are more labile than the cobinamide coenzyme [22].

There are only slight differences in the absorption spectra (Fig. 2a) of aqueous solutions of Co-5′-dCyd-DMBIA-cobamide (II) and Co-5′-dUrd-DMBIA-cobamide (III) when compared with that of coenzyme B_{12} (I). Their main maxima in the region of 260 mμ are shifted toward longer wavelengths and are at 267 and 264 mμ, respectively. Less distinct absorption peaks are also present in the region of 375 mμ and in the visible part of the spectrum at 525 mμ. Also the absorption spectra of

Table 2

Photolysis of nucleoside Co-derivatives of corrinoids

Aqueous solutions of the derivatives (about 3.2×10^{-5} M concn.) were irradiated in silica cells (length 10 mm.) with 60 watt tungsten lamp from a distance of 25 cm. The rate of degradation was calculated from the increase in extinction at 348 mμ. For complete degradation of the analogues the samples were exposed to direct sun-light for 30 min.

No.	Compound	Degradation (%)	
		after 5 min.	after 10 min.
I	Co-5′-dAdo-DMBIA-cobamide	26.1	68.0
IV	Co-5′-dAdo-cobinamide	11.7	48.0
VII	Co-5′-dAdo-TMBIA-cobamide	7.0	28.0
II	Co-5′-dCyd-DMBIA-cobamide	25.4	67.3
V	Co-5′-dCyd-cobinamide	4.2	16.1
VIII	Co-5′-dCyd-TMBIA-cobamide	4.0	15.0
III	Co-5′-dUrd-DMBIA-cobamide	26.1	68.0
VI	Co-5′-dUrd-cobinamide	3.8	14.2
IX	Co-5′-dUrd-TMBIA-cobamide	4.3	16.3

the cytidine (Fig. 2b) and uridine analogues of cobinamide are nearly the same as that of cobinamide coenzyme with the maxima at 264, 375, 380 and 460 mμ. A great similarity to the absorption spectrum of cobinamide coenzyme is exhibited also by the spectra of the Co-5′--deoxynucleoside derivatives of TMBIA-cobamide (Fig. 2c). As it was

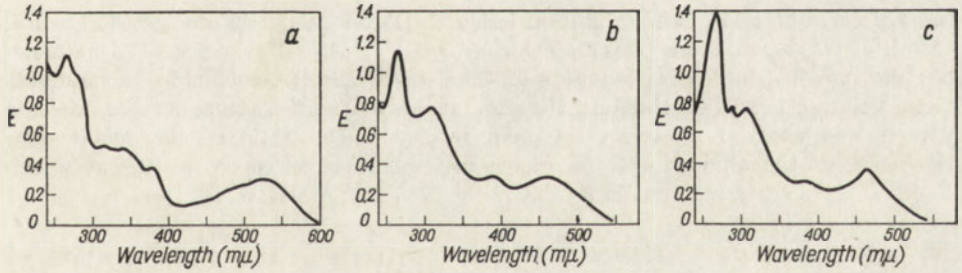


Fig. 2. Absorption spectra of corrin coenzymes in aqueous solution; (*a*), Co-5′--dCyd-DMBIA-cobamide, concn. 3.61×10^{-5} M; (*b*), Co-5′-dCyd-cobinamide, concn. 2.29×10^{-5} M; (*c*), Co-5′-dAdo-TMBIA-cobamide, concn. 3.27×10^{-5} M.

demonstrated by Friedrich & Bernhauer [7], also TMBIA-cobamide cyanide, in spite of the presence of benzimidazole, has optical and electrophoretic properties similar to those of cobinamide cyanide. The appearance of an additional peak at 288 mμ in the spectrum of TMBIA--cobamide is due to the presence of the benzimidazole moiety.

The effect of analogues of the corrin coenzymes on the enzymic conversion of glycerol into β-hydroxypropionaldehyde

In the previous paper from this laboratory [22] the utility of the coenzyme B_{12} dependent reaction of conversion of glycerol into β-hydroxypropionaldehyde, for studying synthetic analogues of coenzyme B_{12} has been demonstrated. It has been also assumed that the reaction proceeds in two steps [17] similarly as the conversion of ethylene glycol into acetaldehyde [24]. In the presence of an excess of enzyme(s) the rate of formation of β-hydroxypropionaldehyde is the function of the concentration of coenzyme B_{12}, within a certain range of its concentration. Under these conditions the effect of the analogue on the reaction could be studied. The effect of Co-5′-dUrd-DMBIA-cobamide (III) is presented in Fig. 3; the analogue inhibited the enzymic reaction, and

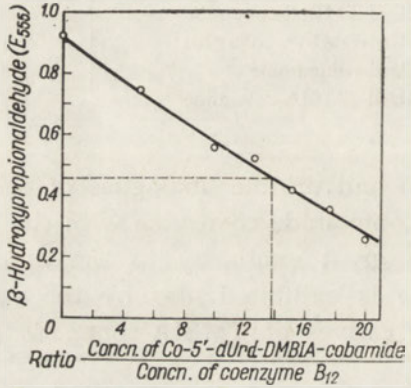


Fig. 3. Determination of the inhibition index (I_{50}). Conditions: 45 μmoles of glycerol; 0.2 ml. of 0.2 M-potassium phosphate buffer, pH 8.0; 500 μg. protein; 0.025 mμmoles of coenzyme B_{12}; increasing amounts of Co-5′-dUrd-DMBIA-cobamide, in mμmoles. Final volume 1 ml. Incubation: 10 min. at 37°. The β-hydroxypropionaldehyde formed was assayed by the tryptophan test [22]. The ratio of the molar concentration of the analogue to the molar concentration of coenzyme B_{12} at which the reaction is inhibited by 50% is defined as I_{50}.

the effect was proportional to its concentration. The concentration of the analogue at which the rate of the reaction is decreased by half can also be found from the diagram. The concentration ratio of the analogue to coenzyme B_{12} at this point is defined as the inhibition index, I_{50}. The results presented in Table 3 show that only cytidyl and uridyl analogues of coenzyme B_{12} inhibit the conversion of glycerol into β-hydroxypropionaldehyde. It was also found (unpublished results) that the inhibition is of a competitive type. On the other hand, the respective analogues of cobinamide coenzyme have almost no inhibitory effect. Only at a concentration 700 times higher than that of coenzyme B_{12} the Co-5′-dUrd--cobinamide (VI) inhibited the rate of the reaction by 50%. Similarly

as the cobinamide coenzyme and its analogues, the analogues of TMBIA--cobamide were without effect on the conversion of glycerol. In these compounds, as a result of methylation of the nitrogen $N_{(3)}$ in benzimidazole, the coordination bond between this atom and the cobalt is cleaved.

Table 3

Effect of synthetic Co-derivatives of corrinoids on enzymic conversion of glycerol into β-hydroxypropionaldehyde

I_{50} is the ratio of the molar concentration of the analogue to the molar concentration of coenzyme B_{12}, at which the reaction is inhibited by 50%. Conditions as in the experiment shown in Fig. 3.

No.	Compound	Inhibition of the reaction (I_{50})
I	Co-5′-dAdo-DMBIA-cobamide	active as coenzyme
IV	Co-5′-dAdo-cobinamide	no effect
VII	Co-5′-dAdo-TMBIA-cobamide	550–600
II	Co-5′-dCyd-DMBIA-cobamide	20
V	Co-5′-dCyd-cobinamide	no effect
VIII	Co-5′-dCyd-TMBIA-cobamide	no effect
III	Co-5′-dUrd-DMBIA-cobamide	14
VI	Co-5′-dUrd-cobinamide	>700
IX	Co-5′-dUrd-TMBIA-cobamide	>700
	Co-methyl-DMBIA-cobamide	1
	Co-methyl-cobinamide	77

Table 4

Conversion of 1,2-diols into deoxyaldehydes by the enzymic system from Aerobacter aerogenes *in the presence of various corrin coenzymes*

Aerobacter aerogenes, PZH nr. 572 was used. Conditions: 45 μmoles of 1,2 diol; 0.2 ml. of 0.2 M-potassium phosphate buffer, pH 8.0; 500 μg. of protein; about 0.1 μmole of a given coenzyme, in a final volume of 1 ml. were incubated for 10 min. at 37°. When glycerol was used as substrate, the tryptophan test was employed [22], and the test with 2,4-dinitrophenylhydrazine according to Böhme & Winkler [4] when ethylene glycol or propanediol were used. (+++), Very strong activity of coenzyme; (—), lack of activity.

Coenzyme	1,2-Diol		
	glycerol	ethylene glycol	propanediol-1,2
Co-5′-dAdo-DMBIA-cobamide	+++	+++	+++
Co-5′-dAdo-cobinamide	–	–	++
Co-5′-dAdo-*a,b,c,d,e,g*-hexamide cobirinic acid	–	–	±
Co-5′-dAdo-α-adenyl-cobamide	+++	+++	+++
Co-5′-dAdo-TMBIA-cobamide	–	–	–

All the data presented here suggest that nucleoside analogues of corrin coenzymes have a smaller inhibiting effect than the alkyl analogues, and that in both groups of compounds the analogues of coenzyme B_{12} are stronger inhibitors than the analogues of cobinamide [22].

It is of interest that among the naturally occurring corrin coenzymes only coenzyme B_{12} and coenzyme pseudo-B_{12} were active in the enzymic system converting glycerol into β-hydroxypropionaldehyde (Table 4). Another reaction catalysed by the same enzymic system, the conversion of ethylene glycol into acetaldehyde, gave similar results. On the other hand, the formation of propionaldehyde from propan-1,2-diol occurred also in the presence of cobinamide coenzyme and, although in traces, it was observed even in the presence of the coenzyme form of hexamide-cobyrinic acid. This was a surprising finding, as it might have been expected that all these reactions proceed in two steps according to the scheme:

$$R - CHOH - CH_2OH \xrightarrow{1} \xrightarrow{2} R - CH_2 - CHO$$

where R = H, CH_3, or CH_2OH, and the arrows denote the two steps of the reaction.

It can be suggested that in the enzymic conversion of glycerol into β-hydroxypropionaldehyde the coordinative bond between cobalt and the nitrogen of imidazole (of benzimidazole or of purine) may play an important role in the activity of the corrin coenzyme. The lack of this bond, as in the cobinamide coenzyme, or its blocking, as e.g. in Co-5′--dAdo-TMBIA-cobamide, deprive the compound of coenzymic properties. However, the differences observed in the activity of the natural corrin coenzymes in the three different reactions of conversion of diols into deoxyaldehydes do not permit of generalization, until further experiments provide more data.

The authors express their gratitude to Prof. Dr. A. DiMarco from Research Laboratory, Farmitalie Milan (Italy) for supplying the strain *Nocardia rugosa* no. 466.

SUMMARY

The synthesis and properties of several nucleoside analogues of corrin coenzymes are described. The effect of the analogues on the enzymic conversion of glycerol into β-hydroxypropionaldehyde was also studied. The reaction was strongly competitively inhibited by the cytidyl (Co-5′-dCyd-DMBIA-cobamide) and uridyl (Co-5′-dUrd-DMBIA-cobamide) analogues. The analogues of cobinamide and of TMBIA-cobamide were without effect or only slightly inhibited the reaction. Differences were also found in the activity of some of the naturally occurring corrin coenzymes in three enzymic reactions converting the diols into aldehydes.

REFERENCES

[1] Barker H. A., Smyth R. D., Weissbach H., Tochey J. I., Ladd J. N. & Volcani B. E. - *J. Biol. Chem.* **235**, 480, 1960.
[2] Bartosiński B., *Enzymatyczna synteza światłoczułych form korynoidów.* Thesis. College of Agriculture, Poznań, 1962.
[3] Bernhauer K., Müller O. & Müller G. - *Biochem. Z.* **336**, 102, 1962.
[4] Böhme H. & Winkler O. - *Z. anal. Chem.* **142**, 1, 1954.
[5] Chambers R. W., Shapiro P. & Kurkov V. - *J. Am. Chem. Soc.* **82**, 970, 1960.
[6] Clark V. M., Todd A. R. & Zussman J. - *J. Chem. Soc.* p. 2952, 1951.
[7] Friedrich W. & Bernhauer K. - *Chem. Ber.* **89**, 2031, 1956.
[8] Friedrich W. & Bernhauer K. - *Chem. Ber.* **89**, 2507, 1956.
[9] Hampton A. - *J. Am. Chem. Soc.* **83**, 3640, 1961.
[10] Hampton A. & Magrath D. I. - *J. Am. Chem. Soc.* **79**, 3250, 1957.
[11] Ikehara M., Ueda T. & Ikeda K. - *Chem. Pharm. Bull.* (Tokyo) **10**, 767, 1962.
[12] Ladd J. N., Hogenkamp H. P. C. & Barker H. A. - *Biochem. Biophys. Res. Comm.* **2**, 143, 1960.
[13] Lenhert P. C. & Hodgkin D. C. - *Nature* **192**, 937, 1961.
[14] Levene P. A. & Tipson R. S. - *J. Biol. Chem.* **106**, 113, 1934.
[15] Migliacci A. & Rusconi A. - *Biochim. Biophys. Acta* **50**, 370, 1961.
[16] Müller O. & Müller G. - *Biochem. Z.* **336**, 299, 1962.
[17] Pawełkiewicz J. & Zagalak B. - *Ann. New York Acad. Sci.*, in press, 1963.
[18] Pawełkiewicz J. & Zodrow K. - *Acta Biochim. Polon.* **4**, 203, 1957.
[19] Sakami W. & Stevens A. - *Bull. Soc. Chim. Biol.* **40**, 1787, 1958.
[20] Smiley K. L. & Sobolov M. - *Arch. Biochem. Biophys.* **97**, 538, 1962.
[21] Smith E. L., Mervyn L., Johnson A. W. & Shaw N. - *Nature* **194**, 1175, 1962.
[22] Zagalak B. - *Acta Biochim. Polon.* **10**, 387, 1963.
[23] Zagalak B. & Pawełkiewicz J. - *Acta Biochim. Polon.* **9**, 315, 1962.
[24] Zagalak B. & Pawełkiewicz J. - *Life Sciences* No. **8**, 395, 1962.

SYNTEZA I WŁASNOŚCI ANALOGÓW KOENZYMÓW KORYNOWYCH

Streszczenie

Opisano syntezę i własności kilku nukleozydowych analogów koenzymów korynowych oraz ich wpływ na zależną od koenzymu B_{12} enzymatyczną reakcję przemiany glicerolu w aldehyd β-hydroksypropionowy. Z syntetyzowanych związków analogi cytydylowy (Co-5′-dCyd-DMBIA-cobamide) i urydylowy (Co-5′-dUrd-DMBIA-cobamide) silnie kompetytywnie hamowały badaną reakcję. Analogi kobinamidu i TMBIA-kobamidu były bez wpływu lub tylko nieznacznie hamowały powyższą przemianę. Stwierdzono także różnice w aktywności kilku naturalnie występujących koenzymów korynowych w trzech enzymatycznych reakcjach przekształcania 1,2-dioli w dezoksyaldehydy.

Received 31 July 1963

Note added in proof: For some enzyme preparations Co-5′-dAdo--TMBIA-cobamide and Co-5′-dAdo-cobinamide proved to be active as coenzymes in the conversion of propanediol into propionaldehyde as well as of ethylene glycol into acetaldehyde.

ACTA BIOCHIMICA POLONICA

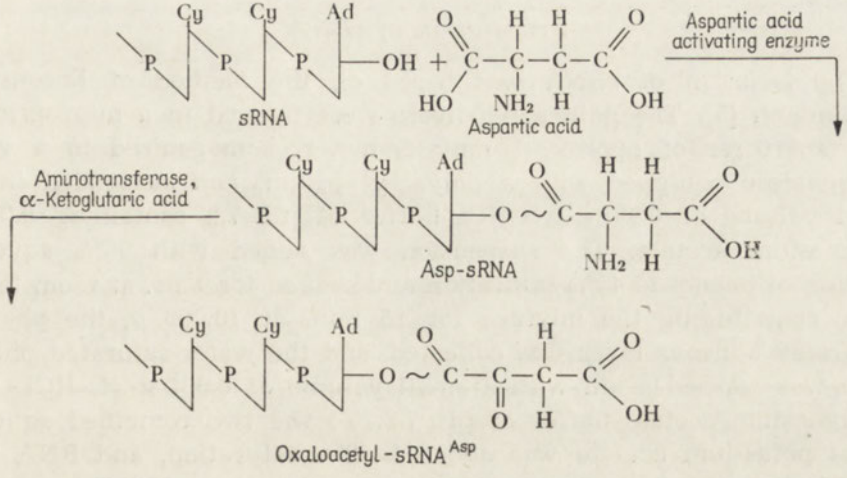
Vol. XI 1964 No. 1

P. SZAFRAŃSKI and S. KLITA

SOLUBLE RIBONUCLEIC ACID AND POLYMERIZATION OF AMINO ACIDS

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa

It is generally assumed that the sequence of amino acids in the polypeptide chain is dependent on the sequence of bases in nucleic acid. Before taking their proper place in the protein molecule, amino acids are first activated and transferred to soluble ribonucleic acid (sRNA), this step being catalysed by activating enzymes which are specific for each amino acid. Apparently, the structural specificity of the amino acid ceases at this step and in the next reaction the nucleotide sequence of sRNA plays a role. The amino acyl-sRNA reacts with the messenger RNA (mRNA) by means of their corresponding bases. According to Crick, corresponding nucleotides of the nucleic acids take part in the reaction and the position of an amino acid in a polypeptide is not dependent directly on its structure. This theory is substantiated by Chapeville *et al.* [4] who synthetized CySH-sRNA and then reduced cysteine to alanine by means of Raney nickel. The compound Ala-sRNA^{CySH} thus



Scheme 1

obtained reacted with poly-UG, which stimulates the incorporation of cysteine, but not of alanine, into polypeptides.

In our experiments, by transamination of aspartic acid combined with sRNA we obtained a mixed hybrid, oxaloacetyl-sRNA^{Asp} (the steps of this reaction are shown in scheme 1), and its effect on the incorporation of amino acids into protein was next studied. It was expected that this compound, which does not possess an amino group, would block protein biosynthesis.

EXPERIMENTAL

Special reagents. The following reagents were employed: guanosinetriphosphate, GTP (Pabst Laboratory, Milwaukee, U.S.A.); reduced glutathione, GSH (Schwarz Bioresearch Inc., New York, U.S.A.); pyridoxal phosphate (Fluka, Switzerland); α-ketoglutaric acid (Nutritional Biochemicals Corp., Cleveland, U.S.A.); reduced nicotinamide-adenine dinucleotide, $NADH_2$ (Reanal, Budapest, Hungary); sodium deoxycholate (Xenon, Łódź, Poland); uniformly ^{14}C-labelled L-leucine (Radiochemical Centre, Amersham, England); [1-^{14}C]L-glycine (Soviet Union); non radioactive amino acids: L-glutamate (Schwarz Lab., U.S.A.); L-histidine (Laokoon, Lvov, Soviet Union); DL-isoleucine (Toscat, England); L-lysine (LaRoche, Switzerland); DL-methionine (Fluka, Switzerland); DL-serine (Chempol, Czechoslovakia); DL-threonine (B. D. H., England); L-aspartic acid (Riedel, Germany); DL-valine (Katowicka Hurtownia Farmaceutyczna, Katowice, Poland); DL-alanine, L-aspartate, L-cysteine, L-hydroxyproline, DL-phenylalanine, L-proline, L-tryptophan, DL-tyrosine and L-glutamic acid (Fabryka Odczynników Chemicznych, Gliwice, Poland). Sodium adenosinetriphosphate (ATP), sodium phosphoenolpyruvate, pyruvic kinase and tris buffer were obtained from the Sigma Chem. Co., St. Louis, U.S.A.

Preparation of sRNA

The isolation of sRNA was based on the method of Ehrenstein & Lipmann [5]. The guinea pig livers were minced in a meat grinder with apertures of approx. 1 mm. diameter, homogenized in a glass homogenizer equipped with a polyacryl piston, and suspended in an equal volume of 0.001 M-tris-HCl buffer of pH 7.2 containing 0.01 M-magnesium acetate. The suspension was added with 90% aqueous solution of phenol to 45% saturation and shaken for 1 hr. at room temp. After centrifuging the mixture for 15 min. at 10 000 *g*, the phenol-saturated aqueous layer was collected, and the water-saturated phenol layer was washed again with a small volume of 0.001 M-tris-HCl - 0.01 M-magnesium acetate buffer of pH 7.2. To the two combined aqueous layers potassium acetate was added to 2% saturation, and RNA was precipitated with two volumes of absolute ethanol at -15°. The precipi-

tate was centrifuged at -5°, dissolved in a small volume of water, then was added with NaCl to 1 M concentration, and centrifuged for 30 min. at 10 000 *g*. The sediment was discarded, and to the supernatant containing sRNA dissolved in 1 M-NaCl, tris in substance was added to pH 8.8. The solution was then incubated for 30 min. at 37° to hydrolyse the amino acyl-sRNA. Next the solution was centrifuged, adjusted to pH 7.5 with 0.1 N-HCl, dialysed overnight against distilled water and lyophilized to dryness. About 80 mg. of sRNA was obtained from 100 g. of liver.

Transfer of aspartic acid to sRNA

The ^{14}C-labelled, and the unlabelled aspartic acid were transferred to sRNA [8] using "pH 5 enzymes" obtained from liver homogenates by a previously described method [11]. The incubation mixture contained per 1 ml.: 20 - 50 mg. sRNA, 3 μmoles ATP, 10.5 μmoles sodium phosphoenolpyruvate, 50 μg. pyruvate kinase, 50 μmoles tris-HCl buffer, pH 7.2, [U-^{14}C]aspartic acid (8.7 μC/μmole), or unlabelled aspartic acid. The mixture was incubated for 10 min. at 37°, an equal volume of 95% aqueous phenol solution was added and Asp-sRNA was isolated as described above, the hydrolysis leading to liberation of amino acids bound with sRNA being omitted. The product was dialysed and its activity was determined with a liquid scintillation counter (SE-1). The activity of [^{14}C]Asp-sRNA was found to be about 2000 counts/mg. sRNA/min.

Transamination of Asp-sRNA

For transamination, the method of Cammarata & Cohen [2] with *α*-ketoglutaric acid, phosphopyridoxal and partially purified aminotransferase from pig heart muscle, was used. The purification of aminotransferase was carried out by the method of Cammarata & Cohen [3], ending at the third step (adjustment of pH of the enzyme solution to 7.5 with 0.1 M-$NaHCO_3$). The details for the transamination are given under Fig. 1. After incubation, to the mixture containing transaminated Asp-sRNA two volumes of absolute ethanol were added in the cold; the precipitate was centrifuged, and a current of cold air was applied to remove the alcohol. The content of oxaloacetyl-$sRNA^{Asp}$ was then determined, the compound being identified by three methods: (1), formation of hydrazones with 2,4-dinitrophenylhydrazine (DNPH); (2), oxidation of $NADH_2$ in the presence of malate dehydrogenase; (3), decarboxylation of [^{14}C]oxaloacetyl-$sRNA^{Asp}$ with simultaneous binding of $^{14}CO_2$ in ethanolamine.

Formation of hydrazones from oxaloacetyl-sRNA^{Asp} and DNPH

The method described by Monier, Stephenson & Zamecnik [7] was used. Approx. 20 mg. of transaminated Asp-sRNA was dissolved in 0.8 ml. water, and 0.2 ml. 0.5 M-acetate buffer of pH 4.0, 0.6 ml. 2-methoxyethanol and 0.8 ml. of 1.2% solution of DNPH in 2-methoxyethanol were added. In some experiments 2-methoxyethanol was replaced by tetrahydrofurfuryl alcohol. The mixture was incubated in the dark for 1 hr. at 37°, then 1.2 ml. distilled water was added, and the excess of DNPH was removed by extracting the solution five times with ethyl acetate. After adding two volumes of absolute alcohol to the solution, sRNA together with DNP-hydrazone of oxaloacetyl-sRNA^{Asp} was centrifuged off. The sediment was dissolved in water, and extinction was determined with a Unicam SP-500 spectrophotometer at 360 mμ.

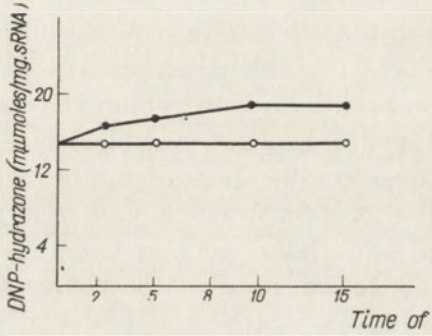


Fig. 1.

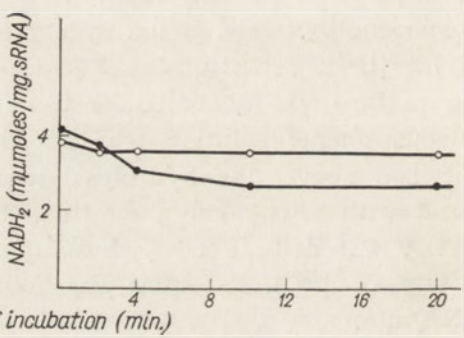


Fig. 2.

Fig. 1. Formation of hydrazone from oxaloacetyl-sRNA^{Asp}. One ml. of the incubation mixture contained: Asp-sRNA, 25 mg.; aminotransferase, 20 μg.; phosphopyridoxal, 25 μg.; α-ketoglutaric acid, 0.2 μmole; and tris-HCl buffer, pH 7.2, 50 μmoles. Final volume 5 ml. Incubation at 37°. At given intervals, samples containing 23 mg. sRNA (measured at 260 mμ) were withdrawn. Cold ethanol was added to the concn. of 66%, the precipitate was centrifuged and dissolved in water. Then DNPH was added and the hydrazones formed were determined at 360 mμ. (●), Proper sample; (○), control, α-ketoglutaric acid omitted, Asp-sRNA not transaminated.

Fig. 2. Oxidation of $NADH_2$ in the presence of oxaloacetyl-sRNA^{Asp} and malate dehydrogenase. One ml. of the incubation mixture contained: Asp-sRNA, 30 mg.; aminotransferase, 8 μg.; malate dehydrogenase, 0.07 μg.; phosphopyridoxal, 10 μg.; $NADH_2$, 21 μg.; tris-HCl buffer, pH 7.2, 50 μmoles. The mixture was preincubated for 30 min. at room temp., cooled to approx 5°, and added with 9.5 μmoles of α-ketoglutaric acid; extinction was read with a spectrophotometer at 340 mμ. The temperature of the mixture was then raised to 37° and the drop in extinction was read at indicated time intervals. The amount of oxidated $NADH_2$ was then calculated in mμmoles per mg. sRNA. (●), Oxidation of $NADH_2$ measured at 340 mμ; (○), control, with aminotransferase inactivated by boiling.

Formation of hydrazone (Fig. 1) in samples containing transaminated Asp-sRNA shows that hybrid oxaloacetyl-sRNA^{Asp} was formed during transamination. Our preparation of oxaloacetyl-sRNA^{Asp} contained a relatively large amount of Asp-sRNA as, under the conditions used, the yield of the transamination even of free aspartic acid was not higher than about 40%.

Oxidation of $NADH_2$ by oxaloacetyl-sRNA^{Asp}

Oxidation of $NADH_2$ was carried out with malate dehydrogenase [10] obtained as described by Ochoa [9]. Pig heart muscle extract was fractionated successively with calcium chloride, ammonium sulphate and ethyl alcohol. Oxidation of $NADH_2$ in the presence of malate dehydrogenase took place simultaneously with the transamination of Asp-sRNA, and the results are illustrated in Fig. 2. Oxidation of $NADH_2$ proves that oxaloacetyl-sRNA^{Asp} is formed from Asp-sRNA.

Decarboxylation of [^{14}C]oxaloacetyl-sRNA^{Asp}

The [^{14}C]Asp-sRNA obtained had an activity ranging from 1200 to 1500 counts/mg. sRNA/min. After transamination, the product was precipitated in the cold with two volumes of absolute ethanol, collected by centrifugation and dissolved in water. Then an amount corresponding to 10 mg. of RNA was transferred to the Warburg vessel. Decarboxylation of oxaloacetyl-sRNA^{Asp} was performed after Krebs [1] in a medium of 0.4 N-H_2SO_4 on a boiling water bath. Under these conditions, no decarboxylation of aspartic acid occurs. The $^{14}CO_2$ formed was absorbed by ethanolamine; although this is not a satisfactory absorbent for CO_2, it was employed because of its low quenching effect. Radioactivity present in ethanolamine was measured with an SE-1 liquid scintillation counter and was found to amount to 300 - 400 counts/min. The presence in ethanolamine of $^{14}CO_2$, which could be derived only from oxaloacetyl-sRNA, is direct evidence of transamination of Asp-sRNA.

Effect of oxaloacetyl-sRNA^{Asp} on the incorporation of amino acids into protein in a cell-free preparation of guinea pig liver

Ribosomes from guinea pig liver were obtained by the method of Lingrel & Webster [6]. Liver tissue, 100 g., was washed, minced and suspended in 0.25 M-saccharose buffered with tris to pH 7.8, and containing added KCl, $KHCO_3$ and $MgCl_2$. The suspension was homogenized for 45 sec. with a polyacryl piston and centrifuged for 15 min. at 18 000 *g*. To the supernatant, sodium deoxycholate was added to 0.5% concentration and the mixture was centrifuged for 1 hr. at 105 000 *g* in a Spinco model L ultracentrifuge. The ribosomes were washed with the same saccharose solution, centrifuged, and suspended

in the same medium for the experiments. The 105 000 *g* supernatant from guinea pig liver was prepared separately without adding deoxycholate. The composition of the incubation mixture containing ribosomes and 105 000 *g* supernatant, used for the incorporation of amino acids is given in Table 1.

Table 1

Effect of oxaloacetyl-sRNA^{Asp} on the incorporation of amino acids into protein

One ml. of the incubation mixture contained: ATP, 3 μmoles; GTP, 0.5 μmole; KCl, 47 μmoles; $MgCl_2$, 5.9 μmoles; sodium phosphoenolpyruvate, 10.5 μmoles; pyruvate kinase, 50 μg.; GSH, 10 μmoles; tris-HCl buffer, pH 7.8, 50 μmoles; [^{14}C]leucine and [^{14}C]glycine, 0.5—0.8 μC (specific activity 5 and 12 μC/μmole, resp.); nonradioactive L-amino acids: aspartate, cysteine, glutamate, glutamic acid, histidine, hydroxyproline, lysine, proline and tryptophan, 0.4 μmole each; and DL-amino acids: alanine, isoleucine, methionine, phenylalanine, serine, threonine, tyrosine and valine, 0.8 μmole each (to the control sample 0.4 μmole of L-aspartic acid was added); ribosomes prepared with deoxycholate from guinea pig liver, 6 mg. of protein; 105 000 *g* supernatant, 3 mg. of protein; sRNA or oxaloacetyl-sRNA^{Asp} as indicated in the Table. Time of incubation was 15 min. at 37°. The reaction was stopped by adding 10% TCA. Protein was washed successively with 5% TCA, with a mixture of alcohol and ether (3:1, by vol.), then with ether, and activity was determined with a liquid scintillation counter SE 1.

Expt. no.	Addition		Counts/mg. protein/min.	% of inhibition
1	sRNA, 1 mg.		124	
	Oxaloacetyl-sRNA^{Asp},	0.5 mg.	107	14
		1.0 mg.	95	23
2	sRNA, 1 mg.		81	
	Oxaloacetyl-sRNA^{Asp},	0.5 mg.	90	0
		1.0 mg.	60	26
		2 mg.	42	49
3	sRNA, 1 mg.		104	
	Oxaloacetyl-sRNA^{Asp},	0.5 mg.	89	14.5
		1 mg.	77	26
		2 mg.	59	44

The data presented indicate an inhibitory effect of oxaloacetyl-sRNA^{Asp} on protein biosynthesis, which increases with rising concentration of oxaloacetyl-sRNA^{Asp} in the sample. Only in one case (expt. 2) the addition of 0.5 mg. of oxaloacetyl-sRNA^{Asp} did not inhibit the incorporation.

Oxaloacetyl-sRNA^{Asp} used in these experiments was not separated from the components of transamination. Control experiments have

Table 2

Effect of the concentration of sRNA and of the components of transamination on the incorporation of amino acids into protein

The composition of the incubation mixture was as described in Table 1, except that the samples contained different amounts of sRNA, and, where indicated, the components of transamination described in Fig. 1 were added. Aminotransferase was inactivated by boiling.

Addition	Counts/mg. protein/min.
sRNA, 1 mg.	55
sRNA, 1 mg., and components of transamination	56
sRNA, 2 mg.	51
sRNA, 2 mg., and components of transamination	50

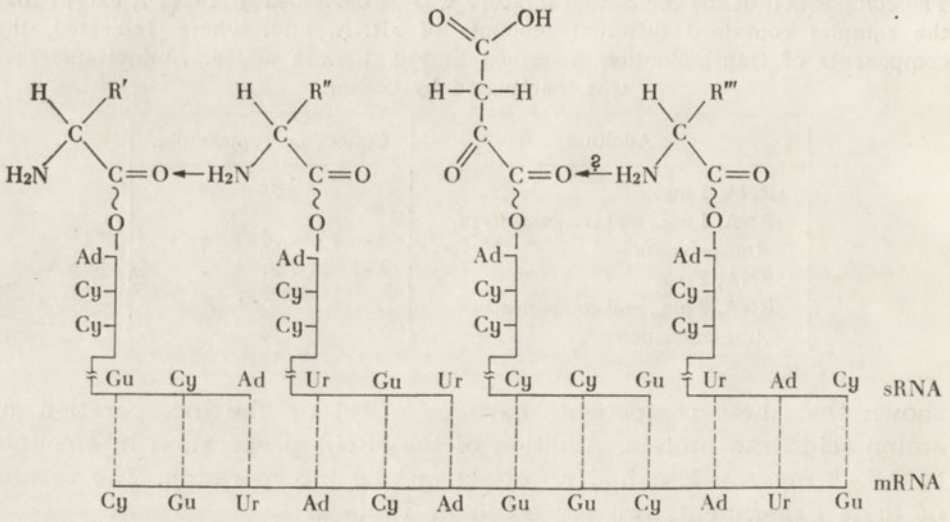
shown that these components have no effect on the incorporation of amino acids into protein. Addition of the sRNA preparation in amounts of 0.5 - 2 mg./ml. also had no effect on the incorporation. The results of these experiments are presented in Table 2.

DISCUSSION

The presented results support the "adaptor" theory of Crick. According to this theory, an amino acid combines with the "adaptor", that is with sRNA which contains a nucleotide sequence for this amino acid. This nucleotide sequence is complementary to the sequence of mRNA, the molecule of which is many times larger than that of sRNA. Hence, various segments of mRNA correspond to different sRNA's, to which the appropriate amino acids are joined. Chapeville *et al.* [4] showed that chemical change of the amino acid connected with sRNA for another amino acid does not change the site of the interaction between sRNA and mRNA. From the work of Takanami [12] it is also known that sRNA free of amino acids binds to ribosomes of *E. coli*. Our experiments suggest that a non-amino acid compound joined to sRNA may also be joined through sRNA to mRNA. The possibility of interaction between oxaloacetyl-sRNA^{Asp} and mRNA is illustrated in scheme 2.

If oxaloacetyl-sRNA^{Asp} which possesses no amino group, joins to mRNA, it should block the formation of the peptide link; in fact, inhibition was observed, although it did not exceed 49% of the control tests. Apparently, considerable amounts of non-transaminated Asp-sRNA present in our preparation of the oxaloacetyl-sRNA^{Asp} prevented the appearance of greater inhibition. On the other hand, the inhibition of protein biosynthesis by oxaloacetyl-sRNA^{Asp} could be dependent on the

position of aspartic acid in the polypeptide chain. If it is present in several places of the chain, formation of peptide links would be prevented, only short peptides soluble in 5% trichloroacetic acid would be formed, and the inhibition would be more effective. If aspartic acid



Scheme 2

is present at the end of the protein molecule, the peptides may be insoluble in trichloroacetic acid and lower inhibition would be observed. It is apparent that complete inhibition by oxaloacetyl-sRNA^{Asp} cannot be expected but the formation of peptides in the reaction inhibited by oxaloacetyl-sRNA^{Asp} could be used for studying the intermediate products of protein synthesis.

It is not yet clear whether oxaloacetic acid, when blocking polypeptide synthesis, combines through its activated carboxyl group with the amino group of the neighbouring amino acid. It is possible that its presence prevents the formation of peptide links. Further studies are needed to elucidate this problem.

This work was supported in part by a grant from the United States Department of Agriculture, Agricultural Research Service.

SUMMARY

Transamination of aspartic acid bound with sRNA was carried out. The product was identified as oxaloacetyl-sRNA^{Asp} by three methods: (1),formation of hydrazones with 2,4-dinitrophenylhydrazine; (2), oxidation of $NADH_2$ in the presence of malate dehydrogenase; (3), decarbo-

xylation of [U-^{14}C]oxaloacetyl-sRNA^{Asp}. The effect of oxaloacetyl-sRNA^{Asp} on the incorporation of amino acids into protein in a cell-free preparation from guinea pig liver was studied; it was found that oxaloacetyl-sRNA^{Asp} possessing no amino group inhibits biosynthesis of protein.

REFERENCES

[1] Arnoff S., in *Techniques of Radiobiochemistry*, p. 145, The Iowa State College Press, Ames, Iowa, USA, 1956.
[2] Cammarata P. C. & Cohen P. P. - *J. Biol. Chem.* **193**, 45, 1951.
[3] Cammarata P. C. & Cohen P. P. - *J. Biol. Chem.* **193**, 53, 1951.
[4] Chapeville F., Lipmann F., von Ehrenstein G., Weisblum B., Ray W. J. & Benzer F. - *Proc. Natl. Acad. Sci. U. S.* **48**, 1086, 1962.
[5] von Ehrenstein G. & Lipmann F. - *Proc. Natl. Acad. Sci. U. S.* **47**, 941, 1961.
[6] Lingrel J. B. & Webster G. - *Biochim. Biophys. Acta* **61**, 942, 1962.
[7] Monier R., Stephenson M. L. & Zamecnik P. C. - *Biochim. Biophys. Acta* **43**, 1, 1960.
[8] Nathans D. & Lipmann F. - *Proc. Natl. Acad. Sci. U. S.* **47**, 497, 1961.
[9] Ochoa S., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.), 1, 735, Acad. Press, New York 1955.
[10] Steinberg D. & Ostrow B. H. - *Proc. Soc. Exptl. Biol. Med.* **89**, 31, 1955.
[11] Szafrański P. & Sułkowski E. - *Acta Biochim. Polon.* **6**, 133, 1959.
[12] Takanami M. - *Biochim. Biophys. Acta* **61**, 432, 1962.

ROZPUSZCZALNY KWAS RYBONUKLEINOWY I POLIMERYZACJA AMINOKWASÓW

Streszczenie

Przeprowadzono transaminację kwasu asparaginowego przyłączonego do sRNA. Uzyskany związek zidentyfikowano jako szczawiooctan-sRNA^{Asp}. Stosowano w tym celu następujące trzy metody: 1) tworzenie się hydrazonów z 2,4-dwunitrofenylohydrazyną; 2) utlenianie $NADH_2$ w obecności dehydrogenazy jabłkowej; 3) dekarboksylacja [^{14}C]szczawiooctanu-sRNA^{Asp} z równoczesnym wiązaniem $^{14}CO_2$ w etanoloaminie. Zbadano następnie wpływ szczawiooctanu-sRNA^{Asp} na włączanie aminokwasów do białka w układzie bezkomórkowym z wątroby świnki morskiej. Wyniki doświadczeń wskazują, że szczawiooctan-sRNA^{Asp}, jako związek pozbawiony grupy aminowej, hamuje biosyntezę białka.

Received 20 September 1963.

ACTA BIOCHIMICA POLONICA

Vol. XI 1964 No. 1

P. SZAFRAŃSKI, JADWIGA LUTOWICZ and LIDIA PUŻYŃSKA

RIBONUCLEIC ACID FROM THE SILK GLAND OF THE SILKWORM AND THE AMINO ACID CODE

Institute of Biochemistry and Biophysics, Polish Academy of Sciences; and Department of Physiological Chemistry, Medical School, Warszawa

In the past few years data have accumulated indicating that biosynthesis of protein molecule is controlled by messenger RNA (mRNA). mRNA is formed in the presence of RNA polymerase and DNA, and its nucleotide composition corresponds to that of DNA. mRNA combines with ribosomes where the proteins are synthesized, becoming the matrix for the synthesis of the appropriate polypeptide. Through mRNA the genetic information is transmitted from DNA to proteins.

Studies on coding of amino acids have led to the discovery that natural [13, 7] and synthetic [3] polynucleotides in cell-free systems can play the role of mRNA. For example, on addition of polyuridylic acid to a cell-free preparation from *E. coli*, polyphenylalanine is formed [8]. Presumably, mRNA from one organism, responsible for the synthesis of a specific protein may also control the synthesis of the same protein in a cell-free system of another organism. For such a type of experiments, RNA from the cells producing a single protein of defined and characteristic amino acid composition should be a suitable material. The silk glands of *Bombyx mori* produce a protein of well-known and highly specific amino acid composition. The posterior part of the silk gland produces fibroin containing 42% glycine and 28% alanine, and the middle part produces sericin, which contains 30% serine.

In this study RNA isolated from the posterior and middle parts of the silk gland of *Bombyx mori* was fractionated, and the nucleotide composition of various fractions was determined. The data thus obtained were compared with the nucleotide composition of mRNA calculated on the basis of the amino acid composition of silk proteins. The effect of RNA from the silk gland on the incorporation of amino acids into proteins in a cell-free preparation from *E. coli* was also studied.

EXPERIMENTAL

Special reagents. The following reagents were used: sodium adenosine triphosphate (ATP), sodium phosphoenolpyruvate, pyruvate kinase, tris buffer, and sodium dodecylsulphate (SDS), Sigma Chemical Co., U.S.A.; sodium guanosinetriphosphate (GTP), Pabst Laboratory, Milwaukee, U.S.A.; reduced glutathione, Schwarz Bioresearch Inc., N.Y., U.S.A.; deoxyribonuclease (DNase), Worthington, U.S.A.; ECTEOLA-cellulose, Brown Co., Berlin; Dowex-1, Serva, Heidelberg; ^{32}P-labelled sodium phosphate was of French origin; [1-^{14}C]glycine, 12.2 μC/μmole, produced in U.S.S.R.; uniformly ^{14}C-labelled amino acids: glycine, 4.5 μC/μmole, Nuclear Chicago Corp., U.S.A.; serine, 3.12 μC/μmole; glutamic acid, 4.3 μC/μmole; valine, 4.76 μC/μmole; and leucine, 5.9 μC/μmole, were received from the Radiochemical Centre, Amersham, England; non-radioactive amino acids: DL-serine, Chempol, Czechoslovakia; L-histidine, Laokoon, Lvov, U.S.S.R.; DL-alanine, L-glutamic acid, glycine, L-leucine, L-tryptophan, DL-tyrosine and DL-valine, F.O.Ch., Gliwice, Poland; β-mercaptoethanol was obtained from the British Drug Houses, London.

Incorporation of ^{32}P into nucleic acids of the silk gland

For the experiments, 20 to 40 caterpillars of the silkworm *Bombyx mori* at the fifth instar, between 4 and 2 days before spinning, were used. The middle and posterior parts of the isolated silk glands were separated and frozen on dry ice.

Five grams of posterior part and the same amount of middle part were washed with 0.01 M-tris buffer, pH 7.2, cut up with scissors, suspended in 3-5 ml. of the same buffer, and homogenized for 2 min. in a glass homogenizer equipped with a polyacryl piston. To the homogenates 150 μC of ^{32}P-labelled Na_2HPO_4 was added, and after 1 min. of incubation at room temperature the mixtures were treated with an equal volume of 90% aqueous solution of phenol containing non-radioactive phosphate and 0.5% sodium dodecyl-sulphate. Then nucleic acids were isolated according to the phenol method of Gierer & Schramm [2]. The preparations were dialysed against water, lyophilized, and digested with 7-10 μg./ml. of DNase at 37° for 1 hr. in 0.01 M-tris buffer of pH 7.0. The solutions of RNA were deproteinized according to Sevag *et al.* [12], dialysed against water, and fractionated on an ECTEOLA-cellulose column.

Fractionation of RNA

Radioactive nucleic acids, 7-10 mg., were applied to the ECTEOLA-cellulose column (0.9 × 20 cm.) previously equilibrated with 0.01 M-tris buffer of pH 7.2. The RNA was eluted with 0.01 M-tris buffer, pH 7.2

(tubes no. 1-30), and then by a gradient from 0 to 1 M-sodium chloride. For this purpose, 3 M buffered NaCl solution and a 200 ml. mixing chamber were used. Fractions of 10 ml. were collected at 2-5 min. intervals, and their absorption was determined at 260 mμ with a Unicam SP-500 spectrophotometer, and radioactivity with a VA-Z VEB Vacutronic counter.

The fractionation of RNA is presented in Figs. 1 and 2. From the middle part of the gland two fractions were obtained. Fraction *I* possessed the highest radioactivity which reached 11 300 counts/mg.RNA/min.

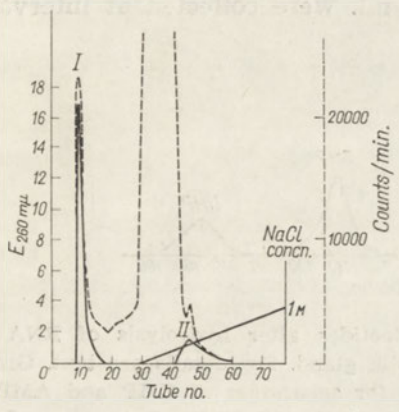


Fig. 1.

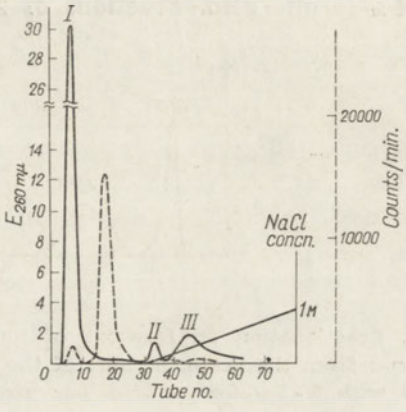


Fig. 2.

Fig. 1. Fractionation of RNA from the middle part of the silk gland of *Bombyx mori*, on ECTEOLA-cellulose column. Beginning with the tube no. 30, gradient NaCl elution was applied. For details see text. (———), Extinction at 260 mμ; (- - -), radioactivity.

Fig. 2. Fractionation of RNA from the posterior part of the silk gland on ECTEOLA-cellulose column. Beginning with tube no. 30, gradient NaCl elution was applied. For details see text. (———), Extinction at 260 mμ; (- - -), radioactivity.

Fraction *II* was contaminated by radioactive inorganic phosphate. When the contamination was subtracted the radioactivity of this fraction appeared to be several times lower than that of fraction *I*. The radioactivity of the three RNA fractions from the posterior part of the gland was many times lower than that of fraction *I* from the middle part.

Determination of the nucleotide composition of RNA

In these experiments 100-200 caterpillars were used. The middle and posterior parts of the silk glands were incubated separately for 5 min. in 0.01 M-tris buffer, pH 7.2, containing 0.5% SDS, and then frozen on dry ice. Moist glands, 50 g., were added with 150 ml. of tris buffer containing SDS and the mixture was homogenized for 1-2 min. at room

temperature. Nucleic acids were isolated by the phenol method, dialysed, lyophilized, digested with DNase, and deproteinized as before. A solution of 15-40 mg. of RNA in 2-6 ml. of water was applied to the ECTEOLA--cellulose column and fractionated as described above. The fractions were concentrated by lyophilization and dialysed. RNA from each fraction, 2-3 mg., was hydrolysed in 0.5 N-KOH at 37° for 20 hr., and after neutralization with perchloric acid, was loaded on a Dowex 1 (0.9 × × 10 cm.) column (formate form).

For the separation of CMP and AMP, gradient elution with from 0 to 1 M-formic acid was used (tubes no. 1-70), and for GMP and UMP, from 1 to 4 M-formic acid. Fractions of 2 ml. were collected at intervals of

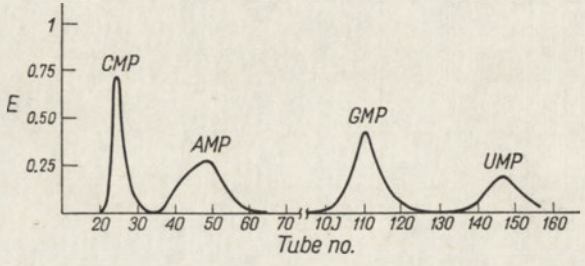


Fig. 3. Fractionation on Dowex 1 of nucleotides after hydrolysis of RNA from fraction *I* from the posterior part of the silk gland. For details see text. Gradient elution with 0 - 1 M-formic acid was used for separation of CMP and AMP, and with 1 - 4 M-formic acid for separation of GMP and UMP. Extinction of CMP was measured at 280 mμ, and that of AMP, GMP and UMP, at 260 mμ.

5-7 min. CMP absorption was measured at 280 mμ, and AMP, GMP and UMP at 260 mμ. The results for fraction *I* from the posterior part of the silk gland are presented in Fig. 3; the results for other fractions were very similar. The amounts of nucleotides were calculated from the millimolar extinction given by Osawa *et al.* [9]. The nucleotide composition of RNA fractions from the silk glands is presented in Table 1.

Table 1

Nucleotide composition of RNA from the silk gland of Bombyx mori

Conditions of hydrolysis and of fractionation of nucleotides are given in the text

Part of silk gland	Fraction from ECTEOLA-cellulose column	Nucleotides			
		CMP	AMP	GMP	UMP
		(molar %)			
Middle	*I*	23.3	23.3	30.1	23.3
	II	24.5	23.3	32.3	19.9
Posterior	*I*	21.3	25.6	32.8	20.3
	II	20.5	23.2	33.9	22.4
	III	22.8	21.9	34.3	21.0

Nucleotide composition of mRNA calculated for silk proteins

Basing on the amino acid composition of silk proteins, the theoretical nucleotide composition of mRNA for sericin and fibroin was calculated. Doublet, triplet and mixed codes were used in the calculations, and the results are presented in Table 2.

Table 2

Nucleotide composition of calculated mRNA for sericin and fibroin

Amino acid composition of silk protein according to Fukuda *et al.* [1]; doublet and mixed codes according to Roberts [10, 11], triplet code according to Wahba *et al.* [14].

Silk protein	Code	Nucleotides			
		CMP	AMP	GMP	UMP
		(molar %)			
Sericin (formed by middle part of the gland)	doublet	25.47	21.99	31.46	21.08
	triplet	16.69	14.92	20.97	47.42
	mixed	22.53	15.64	21.31	40.52
Fibroin (formed by posterior part of the gland)	doublet	20.39	5.18	63.09	11.34
	triplet	13.40	3.65	42.06	40.89
	mixed	15.35	4.97	42.39	37.29

Comparison of the data presented in Tables 1 and 2 indicates that the nucleotide composition of mRNA calculated for sericin according to the doublet code is similar to the composition of RNA from the middle silk glands. On the other hand, there is no similarity between the nucleotide composition of RNA from the posterior glands and the calculated composition of mRNA for fibroin.

Preparation of the cell-free system from E. coli

For the preparation, the method of Nirenberg & Matthaei [8] was used. *E. coli* cells were grown on the medium of Littauer & Kornberg [4] at 37°, harvested by centrifugation, washed with 0.01 M-magnesium acetate - 0.01 M-tris buffer, pH 7.4, and stored at -20°. For the experiments, about 20 g. of the moist cells were ground for 30 min. in a porcelain mortar with 50 g. silica gel at 3-5°. The same temperature was kept during all further steps of the procedure. The mixture was next extracted with 2 volumes of tris - magnesium acetate buffer and was centrifuged at 15 000 *g* for 10 min. The sediment was discarded, to the supernatant β-mercaptoethanol was added to the final concentration of 0.005 M and the mixture was centrifuged at 30 000 *g* for 30 min. The 30 000 *g* supernatant was dialysed overnight against 0.01 M-tris - 0.01 M-

-magnesium acetate - 0.06 M-KCl - 0.005 M-β-mercaptoethanol buffer, pH 7.8. The dialysis residue was concentrated by lyophilization up to about 20 mg. protein/ml. The concentrated 30 000 *g* supernatant containing ribosomes and soluble cytoplasmic proteins was stored at -20°.

In some experiments, the isolated ribosomes and the 105 000 *g* supernatant were used. In this case the *E. coli* cells were broken up by treatment in an ultrasonic disintegrator (18 Kc, 5-10 min., 3-4°), then suspended in tris - magnesium acetate - KCl buffer, pH 7.4, and centrifuged at 20 000 *g* for 20 min. at 0°. The sediment was discarded, and the supernatant was centrifuged at 105 000 *g* for 2 hr. in a Spinco model *L* ultracentrifuge. The supernatant and the sedimented ribosomes suspended in the same buffer were dialysed overnight against 0.01 M-tris - 0.01 M-magnesium acetate - 0.06 M-KCl buffer, pH 7.5, then concentrated by lyophilization and stored at -20°.

Effect of RNA from the silk gland on the incorporation of amino acids into protein in a cell-free preparation from E. coli

In the experiments with RNA from the middle part of the silk gland the labelled amino acids specific for sericin were used; [U-^{14}C]serine, [U-^{14}C]glutamic acid or [1-^{14}C]glycine. For comparison, the incorporation of radioactive leucine and valine, which occur in sericin in minimal amounts, was also studied. In these experiments the 30 000 *g* supernatant of *E. coli* preparation was used.

When the effect of RNA from the posterior part was examined, ribosomes and 105 000 *g* supernatant were used. For the incorporation experiments uniformly labelled [^{14}C]glycine or [^{14}C]serine were applied. The content of these amino acids in fibroin reaches jointly about 57%. The composition of the incubation mixture used in experiments on the incorporation of amino acids is given in Table 3.

The results (Table 3) showed that the addition of RNA from the middle part of the silk gland increased the incorporation of amino acids into proteins in cell-free preparations from *E. coli*. This effect was observed with glycine, glutamic acid and serine, i.e. the amino acids present in sericin in considerable amounts, but not with valine and leucine, which are present in sericin in small amounts (2.8%). Enhanced incorporation of glycine, glutamic acid and serine was observed with fractions *I* and *II* from the ECTEOLA-cellulose column, the intensity of incorporation being greater with fraction *I*. Addition of 0.25 mg. of RNA from fraction *I* per ml. of the incubation mixture caused twofold, or even threefold increase in activity of the synthesized protein. When the amount of RNA was increased to 1 mg., further increase in radioactivity of the protein was observed.

Table 3

Effect of RNA from the middle part of the silk gland on the incorporation of ^{14}C-labelled amino acids into protein in cell-free preparations from E. coli

The incubation mixture: 3 mM-ATP; 10 mM-sodium phosphoenolpyruvate; 30 µg./ml. pyruvate kinase; 0.6 mM-GTP; 10 mM reduced glutathione; 30 mM-KCl; 12 mM-$MgCl_2$; 50 mm-tris buffer, pH 7.5; 30 000 *g* supernatant from *E. coli* containing 10 mg. protein per ml.; sRNA from *E. coli*, 0.5 mg./ml.; ^{14}C-labelled amino acids indicated, 0.5 µC/ml.; non-radioactive L-amino acids: glutamic acid, glycine, histidine, leucine, tryptophan, 0.4 mM each; non-radioactive DL-amino acids: alanine, glycine, serine, tyrosine and valine, 0.8 mM each; RNA from the middle or posterior part of silk gland, 0.25 mg. or 1 mg./ml., except for control samples. Each of the labelled amino acids was applied separately; their specific activities in µC/µmoles: glycine 12.2; glutamic acid, 4.3; serine, 3.12; leucine, 5.9; and valine, 4.76. The samples were incubated for 60 min. at 37°. The reaction was stopped by adding an equal volume of 10% TCA. Protein was washed and heated in 5% TCA for 15 min. at 90°, washed with a mixture of ethanol and ether (3 : 1, by vol.), then with ether, and radioactivity was determined with a liquid scintillation counter, SE 1. Protein was determined according to Lowry *et al.* [5].

Amino acid	Content of amino acid in sericin* (%)	Expt. no.	Control without added RNA	Fraction of RNA from ECTEOLA-cellulose column added			
				I		*II*	
				0.25 mg.	1 mg.	0.25 mg.	1 mg.
			(counts/mg. protein/min.)				
[1-^{14}C]Glycine	8.6	1	100	422	698	270	450
		2	68	192	218	118	
		3	225		438		
[U-^{14}C]Glutamic acid	10.7	1	23	75	143	43	83
		2	15	35	43	23	
		3	29		87		
[U-^{14}C]Serine	30.1	1	320	505	747	370	570
		2	66	176	200	96	
		3	212		446		
[U-^{14}C]Leucine	0.9	1	20	22	19	31	
		2					
		3	32		42		
[U-^{14}C]Valine	1.9	1	15	18	15	20	22
		2	22	31	17	28	
		3	23		31		

* According to Fukuda *et al.* [1].

Experiments performed with RNA from the posterior glands of the silkworm gave entirely different results (Table 4). Total non-fractionated RNA from the posterior glands not only did not stimulate the incorporation of glycine, which is present in fibroin in concentration of 42%,

Table 4

Effect of RNA from the posterior part of the silk gland on the incorporation of ^{14}C-labelled amino acids into protein in cell-free preparations from E. coli

Conditions of experiment as in Table 3. Uniformly labelled [^{14}C]glycine, 4.5 μC/μmole, and serine, 3.12 μC/μmole, were used.

Amino acid	Content of amino acid in fibroin* (%)	Expt. no.	Control without added RNA	With 1 mg. RNA aded
			(counts/mg. protein/min.)	
[U-^{14}C]Glycine	42.8	1	5343	3062
		2	9839	5795
		3	1642	1100
		4	7650	5120
[U-^{14}C]Serine	14.7	1	2343	2291
		2	3600	3543

* According to Fukuda *et al.* [1].

Table 5

Incorporation of amino acids into protein in cell-free preparations from E. coli *incubated with RNA from the middle part of the silk gland*

The amount of amino acids incorporated was calculated in mμmoles fom the results of the experiment presented in Table 3.

Amino acid	Fraction *I* of RNA from ECTEOLA-cellulose column added				Fraction *II* of RNA from ECTEOLA-cellulose colum added			
	0.25 mg.	1 mg.	0.25 mg.	1 mg.	0.25 mg.	1 mg.	0.25 mg.	1 mg.
	calculated		found		calculated		found	
Serine	0.099	0.228	0.099	0.228	0.027	0.133	0.027	0.133
	0.059	0.071	0.059	0.071	0.016		0.016	
		0.125		0.125				
Glycine	0.039	0.091	0.030	0.068	0.011	0.053	0.010	0.034
	0.024	0.028	0.017	0.021	0.0064		0.0068	
		0.050		0.029				
Glutamic acid	0.025	0.058	0.020	0.047	0.007	0.034	0.008	0.023
	0.015	0.018	0.008	0.011	0.004		0.003	
		0.032		0.023				

but inhibited up to 60% the incorporation of this amino acid nto the proteins in *E. coli* cell-free system. The incorporation of serine was not affected by RNA from the posterior glands.

The amount of amino acids which should be incorporated into protein during biosynthesis of sericin was calculated on the basis of he data collected in Table 3 and the amino acid composition of sericin taking

into account the known specific activity values for serine, glycine and glutamic acid. The results were compared with the amounts of amino acids found to be incorporated during the experiments. The amount of incorporated serine was taken as 100%, the incorporation of glycine and glutamic acid being calculated in relation to that of serine. The results presented in Table 5 indicate that the amounts of amino acids incorporated into protein in the cell-free preparation from *E. coli* are similar to those expected to occur in sericin.

DISCUSSION

The posterior and middle parts of silk glands of the silkworm *Bombyx mori* belong to those rare systems which form a single protein. This gives the possibility of isolation of RNA responsible for the synthesis of a defined polypeptide. Separation of RNA on ECTEOLA-cellulose column gave two fractions from the middle part of the silk gland, and three fractions from the posterior one. The obtained fractions of RNA differed in the rate of synthesis measured by the incorporation of ^{32}P, as well as in biological properties. Fraction *I* from the middle part showed the highest turnover rate, and its activity amounted to 11 300 counts/mg.RNA/min. In three separate tubes of this fraction the activity of RNA was similar, indicating the homogeneity of the material. Radioactivity of the second fraction, and of the fractions of RNA from the posterior part of the gland was much lower.

The nucleotide composition of the different fractions of RNA separated on the ECTEOLA-cellulose column was very similar. All the fractions possessed a high content of GMP, reaching 30-34%. The nucleotide composition of mRNA calculated for sericin according to the doublet code is similar to the experimentally obtained composition of RNA isolated from the middle part of the silk gland (Tables 1 and 2). Yet, there is no similarity between the composition of RNA from the posterior part and the nucleotide composition of mRNA calculated for fibroin by means of various codes.

The experiments showed that RNA from the middle silk gland enhanced the incorporation of amino acids specific for sericin, into *E. coli* proteins. Serine, glycine and glutamic acid, which account for about 50% of the composition of sericin, were incorporated in proportions approximately the same as those in which they occur in sericin. Different results were obtained with RNA from the posterior silk gland which inhibited the incorporation of glycine into *E. coli* proteins, in spite of the fact that this amino acid is present in fibroin in a very high amount. A similar inhibition of incorporation of amino acids in the cell-free system from *E. coli* was observed by Möller & Ehrenstein [6] who employed synthetic polynucleotides. It seems that both silkworm RNA

and synthetic polynucleotides combine with the mRNA of ribosomes. In this way mRNA of *E. coli* may be blocked, resulting in inhibition of protein synthesis.

Further experiments will show whether the protein synthesized in the cell-free preparation from *E. coli* under the influence of RNA from the middle part of the silk gland of the silkworm resembles sericin.

We wish to thank Miss Krystyna Derkus for valuable assistance in the experimental work.

SUMMARY

The middle and the posterior parts of the silk gland of the silkworm belong to those rare systems which produce a single protein of a specific amino acid composition. This gives the possibility of isolation of RNA responsible for the synthesis of a defined polypeptide.

The RNA was isolated from the silk gland by the phenol method and fractionated on the ECTEOLA-cellulose column. The nucleotide composition of each RNA fraction was estimated and compared with the composition calculated with the use of doublet, triplet and mixed codes.

The effect of several fractions of RNA from the silk gland on the incorporation of ^{14}C-labelled amino acids into the proteins in cell-free preparations from *E. coli* was studied. The values obtained were compared with the calculated amounts of amino acids which should be incorporated into protein during sericin biosynthesis.

REFERENCES

[1] Fukuda T., Kirimura J., Matuda M. & Suzuki T. - *J. Biochem.*, Tokyo, **42**, 341, 1955.
[2] Gierer A. & Schramm G. - *Nature* **177**, 702, 1956.
[3] Lengyel P., Speyer I. F., Basilio C. & Ochoa S. - *Proc. Natl. Acad. Sci. U.S.* **48**, 282, 1962.
[4] Littauer M. Z. & Kornberg A. - *J. Biol. Chem.* **226**, 1077, 1957.
[5] Lowry D. H., Rosebourgh N. S., Farr A. L. & Rendoli R. S. - *J. Biol. Chem.* **193**, 265, 1951.
[6] Möller W. J. & von Ehrenstein G. - *Biochem. Biophys. Res. Comm.* **11**, 325, 1963.
[7] Nathans D., Notani G., Schwartz J. H. & Zinder N. D. - *Proc. Natl. Acad. Sci. U.S.* **48**, 1424, 1962.
[8] Nirenberg M. W. & Matthaei J. H. - *Proc. Natl. Acad. Sci. U.S.* **47**, 1588, 1961.
[9] Osawa S., Takata K. & Hotta Y. - *Biochim. Biophys. Acta* **28**, 271, 1958.
[10] Roberts R. B. - *Proc. Natl. Acad. Sci. U.S.* **48**, 897, 1962.
[11] Roberts R. B. - *Proc. Natl. Acad. Sci. U.S.* **48**, 1245, 1962.

[12] Sevag M. G., Lackman D. B. & Smolens J. - *J. Biol. Chem.* **124**, 425, 1938.
[13] Tsugita A., Fraenkel-Conrat H., Nirenberg M. W. & Matthaei J. H. - *Proc. Natl. Acad. Sci. U.S.* **48**, 846, 1962.
[14] Wahba A. J., Gardner R. S., Basilio C., Speyer J. F. & Lengyel P. - *Proc. Natl. Acad. Sci. U.S.* **49**, 116, 1963.

KWAS RYBONUKLEINOWY Z GRUCZOŁÓW PRZĘDNYCH JEDWABNIKA I KODOWANIE AMINOKWASÓW

Streszczenie

Tylne i środkowe odcinki gruczołów przędnych jedwabnika morwowego należą do nielicznych układów wytwarzających pojedyncze białka o charakterystycznym składzie aminokwasowym. Stwarza to możliwości wyizolowania z nich RNA odpowiedzialnego za syntezę określonego polipeptydu.

RNA wyizolowano wg. metody fenolowej z gruczołów przędnych i frakcjonowano na kolumnie ECTEOLA-celuloza. Oznaczano skład nukleotydowy poszczególnych frakcji, który porównano ze składem wyliczonym przy użyciu kodu dwójkowego, trójkowego i mieszanego.

Zbadano wpływ poszczególnych frakcji RNA z gruczołów przędnych na włączanie ^{14}C-aminokwasów do białek w bezkomórkowych preparatach z *E. coli*. Otrzymane wyniki porównano z wyliczonymi ilościami aminokwasów, które powinny wbudować się do białka podczas biosyntezy serycyny.

Received 2 October 1963.

ACTA BIOCHIMICA POLONICA

Vol. XI 1964 No. 1

RECENZJE KSIĄŻEK

J. K. Aikagawa: THE ROLE OF MAGNESIUM IN BIOLOGIC PROCESSES. Charles C. Thomas Publ., Springfield (Ill.) 1963; str. 117, cena $ 6.75.

W krótkiej monografii, składającej się z 26 rozdziałów, Autor wprowadza czytelnika w zagadnienie dwoma przyjemnie i żywo napisanymi rozdziałami o przeszło trzechsetletniej historii kariery magnezu, m.in. w czasy odkryć Davy'ego, Grignarda i Willstättera, oraz zapoznaje z pierwszymi doświadczeniami z XIX wieku nad fizjologicznym działaniem soli magnezu. Dalsze rozdziały omawiają zjawiska fizjologiczne i patologiczne, w których obserwuje się zaburzenia przemiany magnezu, oraz przedstawiają aktualny stan wiedzy o hypomagnezemii. W osobnym rozdziale jest mowa o najczęściej stosowanych metodach oznaczania magnezu w materiale biologicznym. Na podstawie własnych doświadczeń Autor uważa metodę przy użyciu molibdenowanadianu za najodpowiedniejszą. Polecana procedura jest jednak pośrednia i żmudna i wydaje się, że w pracowni Autora stosowano ją raczej ze względu na łatwość przygotowania tą drogą próbek do jednoczesnego oznaczenia promieniotwórczego magnezu w doświadczeniach izotopowych. Dobrze nadające się do celów klinicznych proste metody kompleksometryczne zostały omówione zbyt krótko.

Dużą część monografii poświęca Autor badaniom nad ruchem i rozmieszczeniem magnezu w organizmie zwierząt i człowieka. Są to głównie prace własne Autora i jego współpracowników, w tym dużo prac jeszcze niepublikowanych. Zapoczątkowanie tych badań stało się możliwe dopiero w roku 1957 dzięki odkryciu i udostępnieniu izotopu promieniotwórczego magnezu ^{28}Mg o stosunkowo długim okresie połowicznego rozpadu, bo 21,3 godz. Z całej ilości magnezu zawartego w ciele dorosłego człowieka (ok. 25 g.) ok. 60% występuje w kościach, 40% w tkankach miękkich, a tylko ok. 1% w płynach pozakomórkowych organizmu. Ciekawie są przedstawione badania nad wpływem różnych czynników na wychwytywanie magnezu przez tkanki; omówiono zwłaszcza wpływ hormonów oraz inhibitorów procesów metabolicznych. Dla zrozumienia roli magnezu pomocne byłoby szersze omówienie mechanizmów tych procesów biochemicznych, w których magnez odgrywa rolę, jednak zamierzona zwięzłość monografii zapewne na to nie pozwoliła.

Zagadnienia omawiane w książce przedstawiono w sposób bardzo przystępny i żywy, a ich zakres pozwala uzyskać ogólny obraz przedmiotu. Książkę tę można polecić wszystkim, którzy interesują się zagadnieniem gospodarki mineralnej w ustroju.

Tadeusz Chojnacki

E. Chargaff, ESSAYS ON NUCLEIC ACIDS. Elsevier Publishing Co., Amsterdam, London, New York, 1963; p. 211; price Dfl. 22.50, 45/-sh, DM 25.--.

The book gives an account of research on nucleic acids in the last 15 years. Chemical, physical and biological properties of nucleic acids are described as well as the role of the latter in directing the synthesis of specific proteins. These intrinsic problems are presented in a very clear and simple way.

Chapter 1 deals with the chemical specificity of nucleic acids and the mechanism of their enzymic degradation. The Author discusses the identity and diversity in high-molecular cell constitutents, describes the methods of preparation and hydrolysis of nucleic acids and separation of purines and pyrimidines. This section also contains the composition of DNA and RNA and treats about the conditions of their enzymic depolymerization. Chapters 2 to 6 are devoted to physics, chemistry and function of nucleic acids and nucleoproteins. The structure and biological significance of two kinds of nucleic acids as well as nucleoproteins is described. These chapters contain also the methods of fractionation of nucleic acids and discuss the significance of isolated constituents. In chapters 7 and 8 the Author describes the nucleic acids as carriers of genetic information. The problem of protein biosynthesis in relation to nucleic acids is shortly discussed as well as the steps towards a chemistry of heredity. Chapters 9 and 10 sumarize the investigations on nucleotide sequence and the remarks on the coding problem. Chapter 11 containing a discussion on general problems of biochemistry of nucleic acids, closes the book.

Most of the material has been published previously elsewhere and is reprinted in the book without change. Chapter 9 underwent extensive revision, while chapters 10 and 11 have not been published previously.

The book as a whole is a very valuable contribution to our knowledge of nucleic acids.

Przemysław Szafrański

COMPREHENSIVE BIOCHEMISTRY (M. Florkin & E. H. Stotz, eds.). Elsevier Publishing Co., Amsterdam, London, New York, 1963. Vol. 5, Carbohydrates; str. XVI+327, cena Dfl. 40.-, £ 4.-.-, DM 44.50. Vol. 10, Sterols, bile acids and steroids; str. XIII + 209, cena Dfl. 27.50, 55/-sh, DM 31.- -.

Omawiane tomy stanowią część dzieła, które ma za zadanie przedstawić w jednej całości teoretyczny i doświadczalny materiał biochemii. Całość ma objąć około dwudziestu pięciu tomów w pięciu sekcjach, a mianowicie: I, fizyko-chemiczne i organiczne aspekty biochemii; II, chemia związków biologicznych; III, mechanizm reakcji biochemicznych; IV, metabolizm; oraz V, biologia chemiczna. Obydwa recenzowane tomy należą do sekcji drugiej.

Węglowodanom jest poświęcony tom 5, zawierający następujące rozdziały: monosacharydy (E. Percival); kwasy aldonowe, uronowe i askorbinowe (E. Percival); aminocukry (H. Egge); fosforany cukrowców (L. F. Leloir i C. E. Cardini); glikozydy (J. Conchie i G. A. Levvy); oligosacharydy (S. Tsuiki, Y. Hashimoto i W. Pigman); obszerny, podzielony na pięć części rozdział o wielocukrach (D. Horton, M. L. Wolfrom, Z. I. Kertesz, S. A. Barker i R. W. Jeanloz); oraz cyklitole (S. J. Angyal).

Tom 10 poświęcony sterolom, kwasom żółciowym i steroidom opracowali D. Kritchevsky (sterole), G. A. D. Haslewood (kwasy żółciowe), G. I. Fujimoto i R. W. Ledeen (androgeny), P. A. Katzman i W. H. Elliott (estrogeny), H. J. Ringold i A. Bowers (steroidy nadnerczy) oraz J. A. Zderic (progesteron).

Poszczególne rozdziały są opracowane zwięźle, z podaniem piśmiennictwa; zdaniem recenzenta odpowiadają one w pełni temu, czego oczekuje się od opracowania ujmującego całość poszczególnych działów. Obydwa tomy Comprehensive Biochemistry będą w każdej pracowni biochemicznej bardzo pożyteczną książką.

[12] Sevag M. G., Lackman D. B. & Smolens J. - *J. Biol. Chem.* **124**, 425, 1938.
[13] Tsugita A., Fraenkel-Conrat H., Nirenberg M. W. & Matthaei J. H. - *Proc. Natl. Acad. Sci. U.S.* **48**, 846, 1962.
[14] Wahba A. J., Gardner R. S., Basilio C., Speyer J. F. & Lengyel P. - *Proc. Natl. Acad. Sci. U.S.* **49**, 116, 1963.

KWAS RYBONUKLEINOWY Z GRUCZOŁÓW PRZĘDNYCH JEDWABNIKA I KODOWANIE AMINOKWASÓW

Streszczenie

Tylne i środkowe odcinki gruczołów przędnych jedwabnika morwowego należą do nielicznych układów wytwarzających pojedyncze białka o charakterystycznym składzie aminokwasowym. Stwarza to możliwości wyizolowania z nich RNA odpowiedzialnego za syntezę określonego polipeptydu.

RNA wyizolowano wg. metody fenolowej z gruczołów przędnych i frakcjonowano na kolumnie ECTEOLA-celuloza. Oznaczano skład nukleotydowy poszczególnych frakcji, który porównano ze składem wyliczonym przy użyciu kodu dwójkowego, trójkowego i mieszanego.

Zbadano wpływ poszczególnych frakcji RNA z gruczołów przędnych na włączanie ^{14}C-aminokwasów do białek w bezkomórkowych preparatach z *E. coli*. Otrzymane wyniki porównano z wyliczonymi ilościami aminokwasów, które powinny wbudować się do białka podczas biosyntezy serycyny.

Received 2 October 1963.

ACTA BIOCHIMICA POLONICA

Vol. XI 1964 No. 1

RECENZJE KSIĄŻEK

J. K. Aikagawa: THE ROLE OF MAGNESIUM IN BIOLOGIC PROCESSES. Charles C. Thomas Publ., Springfield (Ill.) 1963; str. 117, cena $ 6.75.

W krótkiej monografii, składającej się z 26 rozdziałów, Autor wprowadza czytelnika w zagadnienie dwoma przyjemnie i żywo napisanymi rozdziałami o przeszło trzechsetletniej historii kariery magnezu, m.in. w czasy odkryć Davy'ego, Grignarda i Willstättera, oraz zapoznaje z pierwszymi doświadczeniami z XIX wieku nad fizjologicznym działaniem soli magnezu. Dalsze rozdziały omawiają zjawiska fizjologiczne i patologiczne, w których obserwuje się zaburzenia przemiany magnezu, oraz przedstawiają aktualny stan wiedzy o hypomagnezemii. W osobnym rozdziale jest mowa o najczęściej stosowanych metodach oznaczania magnezu w materiale biologicznym. Na podstawie własnych doświadczeń Autor uważa metodę przy użyciu molibdenowanadianu za najodpowiedniejszą. Polecana procedura jest jednak pośrednia i żmudna i wydaje się, że w pracowni Autora stosowano ją raczej ze względu na łatwość przygotowania tą drogą próbek do jednoczesnego oznaczenia promieniotwórczego magnezu w doświadczeniach izotopowych. Dobrze nadające się do celów klinicznych proste metody kompleksometryczne zostały omówione zbyt krótko.

Dużą część monografii poświęca Autor badaniom nad ruchem i rozmieszczeniem magnezu w organizmie zwierząt i człowieka. Są to głównie prace własne Autora i jego współpracowników, w tym dużo prac jeszcze niepublikowanych. Zapoczątkowanie tych badań stało się możliwe dopiero w roku 1957 dzięki odkryciu i udostępnieniu izotopu promieniotwórczego magnezu ^{28}Mg o stosunkowo długim okresie połowicznego rozpadu, bo 21,3 godz. Z całej ilości magnezu zawartego w ciele dorosłego człowieka (ok. 25 g.) ok. 60% występuje w kościach, 40% w tkankach miękkich, a tylko ok. 1% w płynach pozakomórkowych organizmu. Ciekawie są przedstawione badania nad wpływem różnych czynników na wychwytywanie magnezu przez tkanki; omówiono zwłaszcza wpływ hormonów oraz inhibitorów procesów metabolicznych. Dla zrozumienia roli magnezu pomocne byłoby szersze omówienie mechanizmów tych procesów biochemicznych, w których magnez odgrywa rolę, jednak zamierzona zwięzłość monografii zapewne na to nie pozwoliła.

Zagadnienia omawiane w książce przedstawiono w sposób bardzo przystępny i żywy, a ich zakres pozwala uzyskać ogólny obraz przedmiotu. Książkę tę można polecić wszystkim, którzy interesują się zagadnieniem gospodarki mineralnej w ustroju.

Tadeusz Chojnacki

E. Chargaff, ESSAYS ON NUCLEIC ACIDS. Elsevier Publishing Co., Amsterdam, London, New York, 1963; p. 211; price Dfl. 22.50, 45/-sh, DM 25.--.

The book gives an account of research on nucleic acids in the last 15 years. Chemical, physical and biological properties of nucleic acids are described as well as the role of the latter in directing the synthesis of specific proteins. These intrinsic problems are presented in a very clear and simple way.

Chapter 1 deals with the chemical specificity of nucleic acids and the mechanism of their enzymic degradation. The Author discusses the identity and diversity in high-molecular cell constitutents, describes the methods of preparation and hydrolysis of nucleic acids and separation of purines and pyrimidines. This section also contains the composition of DNA and RNA and treats about the conditions of their enzymic depolymerization. Chapters 2 to 6 are devoted to physics, chemistry and function of nucleic acids and nucleoproteins. The structure and biological significance of two kinds of nucleic acids as well as nucleoproteins is described. These chapters contain also the methods of fractionation of nucleic acids and discuss the significance of isolated constituents. In chapters 7 and 8 the Author describes the nucleic acids as carriers of genetic information. The problem of protein biosynthesis in relation to nucleic acids is shortly discussed as well as the steps towards a chemistry of heredity. Chapters 9 and 10 sumarize the investigations on nucleotide sequence and the remarks on the coding problem. Chapter 11 containing a discussion on general problems of biochemistry of nucleic acids, closes the book.

Most of the material has been published previously elsewhere and is reprinted in the book without change. Chapter 9 underwent extensive revision, while chapters 10 and 11 have not been published previously.

The book as a whole is a very valuable contribution to our knowledge of nucleic acids.

Przemysław Szafrański

COMPREHENSIVE BIOCHEMISTRY (M. Florkin & E. H. Stotz, eds.). Elsevier Publishing Co., Amsterdam, London, New York, 1963. Vol. 5, Carbohydrates; str. XVI+327, cena Dfl. 40.-, £ 4.-.-, DM 44.50. Vol. 10, Sterols, bile acids and steroids; str. XIII + 209, cena Dfl. 27.50, 55/-sh, DM 31.- -.

Omawiane tomy stanowią część dzieła, które ma za zadanie przedstawić w jednej całości teoretyczny i doświadczalny materiał biochemii. Całość ma objąć około dwudziestu pięciu tomów w pięciu sekcjach, a mianowicie: I, fizyko-chemiczne i organiczne aspekty biochemii; II, chemia związków biologicznych; III, mechanizm reakcji biochemicznych; IV, metabolizm; oraz V, biologia chemiczna. Obydwa recenzowane tomy należą do sekcji drugiej.

Węglowodanom jest poświęcony tom 5, zawierający następujące rozdziały: monosacharydy (E. Percival); kwasy aldonowe, uronowe i askorbinowe (E. Percival); aminocukry (H. Egge); fosforany cukrowców (L. F. Leloir i C. E. Cardini); glikozydy (J. Conchie i G. A. Levvy); oligosacharydy (S. Tsuiki, Y. Hashimoto i W. Pigman); obszerny, podzielony na pięć części rozdział o wielocukrach (D. Horton, M. L. Wolfrom, Z. I. Kertesz, S. A. Barker i R. W. Jeanloz); oraz cyklitole (S. J. Angyal).

Tom 10 poświęcony sterolom, kwasom żółciowym i steroidom opracowali D. Kritchevsky (sterole), G. A. D. Haslewood (kwasy żółciowe), G. I. Fujimoto i R. W. Ledeen (androgeny), P. A. Katzman i W. H. Elliott (estrogeny), H. J. Ringold i A. Bowers (steroidy nadnerczy) oraz J. A. Zderic (progesteron).

Poszczególne rozdziały są opracowane zwięźle, z podaniem piśmiennictwa; zdaniem recenzenta odpowiadają one w pełni temu, czego oczekuje się od opracowania ujmującego całość poszczególnych działów. Obydwa tomy Comprehensive Biochemistry będą w każdej pracowni biochemicznej bardzo pożyteczną książką.

Na szczególne podkreślenie zasługuje piękne wydanie książki. Znakomity papier, bardzo staranny dobór czcionek, dobrze wykonana i piękna oprawa oraz poręczna całość książki wskazują na to, że kilkusetletnia tradycja domu wydawniczego Elsevier nie poszła w zapomnienie.

Włodzimierz Mozołowski

I. Sunshine and S. R. Gerber, SPECTROPHOTOMETRIC ANALYSIS OF DRUGS INCLUDING ATLAS OF SPECTRA. Charles C. Thomas Publisher, Springfield (Ill.) 1963; stron xvii+235, cena $ 10.50.

Rozwój techniki spektrofotometrycznych metod analizy stworzył możliwość stosunkowo szybkiej i mało skomplikowanej identyfikacji związków chemicznych, wykazujących charakterystyczne pasma absorpcji światła pozafioletowego, widzialnego lub podczerwonego. Zastosowanie samorejestrujących spektrofotometrów do ultrafioletu i podczerwieni umożliwia identyfikację również wielu leków zarówno w czystych roztworach jak i w materiale biologicznym pod warunkiem, że dysponuje się odpowiednim atlasem widm absorpcyjnych wykonanych w różnych warunkach. Trud sporządzenia takiego atlasu podjęli Autorzy omawianej książki. Zawiera ona spektra stu pięćdziesięciu leków, których identyfikacja może mieć znaczenie w praktyce klinicznej lub sądowo-lekarskiej. Dla większości leków podano wykresy widm zarówno w świetle pozafioletowym, jak i w podczerwieni. Widmo każdego związku w ultrafiolecie podano dla roztworów (ekstraktów) kwaśnych i zasadowych danego związku. Spektra w podczerwieni podane są dla próbek rozpuszczonych w chloroformie, jak i dla próbek sprasowanych ze stałym bromkiem potasu.

Atlas poprzedzony jest opisem sposobu ekstrakcji krwi w celu badania widma w ultrafiolecie oraz opisem przygotowania próbek do badania w zakresie podczerwonej części widma. Szczególnie cenną częścią książki jest indeks nazw leków opisanych w atlasie, który to indeks zawiera wiele synonimów nazw powszechnie używanych. Wydaje się, że wykaz umieszczony na początku książki, ułożony według kolejności długości fali, w której wypada maksimum pochłaniania światła pozafioletowego, ułatwi praktyczne korzystanie z książki w celu identyfikacji leków zawartych w krwi.

Zalecany w omawianej książce sposób spektrofotometrycznej identyfikacji leków na pewno nie jest uniwersalny i nie do wszystkich związków da się zastosować. Jednakże tam, gdzie badany związek ma charakterystyczne widmo, polecany sposób pozwala na pominięcie trudów związanych z chemiczną analizą.

Z tego względu książka będzie pożyteczną pomocą nie tylko dla laboratoriów klinicznych i sądowo lekarskich, ale także dla wszystkich pracowni analitycznych zajmujących się oznaczaniem w materiale biologicznym związków chemicznych obcych ustrojowi żywemu. Oczywiście wtedy, kiedy będą one dysponowały nie tylko książką, ale także odpowiednimi spektrofotometrami.

Mariusz Żydowo