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EVALUATION OF METALLOTHIONEIN CONTENT IN ANIMAL TISSUES

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A method has been proposed for approximate estimation of metallothionein in rat kidney and liver. The method is based on tracing metallothionein with active mercury and subsequent measurement of mercury content in homogenates deproteinized with trichloroacetic acid. The amount of bound mercury is proportional to the concentration of metallothionein in the tissue; internal standards of metallothionein have been applied in the method. Preliminary determinations have shown that the content of metallothionein in the tissues of healthy rat amounts to about 0.1 mg/g (liver) and 0.4 mg/g (kidney), whereas in rats exposed to cadmium the respective values were 4.4 mg/g and 4.1 mg/g.

Metallothionein, a low-molecular-weight protein of high SH content, exhibits remarkable ability for binding cadmium and mercury in animal tissues. The occurrence of this protein and its complexes with cadmium or mercury in kidney and liver was proved in the following species: horse (Kägi & Vallee, 1960, 1961), rabbit (Piscator, 1964), rat (Wiśniewska *et al.*, 1970) and man (Pulido *et al.*, 1966; Wiśniewska-Knypl *et al.*, 1971). The content of metallothionein in various tissues is probably subject to large changes (Piscator, 1964).

Analytical methods for determination of metallothionein in animal tissues have not so far been described. The elaborated procedure was based on separation of low-molecular metallothionein complexed with mercury from other metal-binding proteins. Determination of mercury in the separated fraction allows estimation of metallothionein since no appreciable amounts of other low-molecular-weight compounds capable of chelating mercury, are present in this fraction.

A preliminary report has been presented at the IX Meeting of the Polish Biochemical Society, Katowice, September 8 - 11, 1971.

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EXPERIMENTAL

Materials

Metallothionein used as an internal standard in the analytical assay, was prepared from the equine renal cortex according to Pulido *et al.* (1966). The preparation had the following properties: mol. wt. 10 400 (by gel filtration on Sephadex G-75); ε at 250 nm at pH 7.0, 6.5×10^4 litre$\cdot$mol$^{-1}\cdot$cm^{-1}; Cd content 2.6%; Zn content 1.6% (determined colorimetrically with dithizone after Elkins, 1959); SH groups, 980 μmol$\cdot$g^{-1} (determined colorimetrically with HEDD[1] after Bitny-Szlachto *et al.*, 1963). These properties were within the limits reported earlier by Kägi & Vallee (1960, 1961), with the SH groups content at the lower limit. A control chromatography of the preparation on DEAE-cellulose in a stepwise concentration gradient of tris-HCl buffer, pH 8.6, did not reveal any substantial contamination of the preparation. Standard solutions, 1 mg of metallothionein per 1 ml of water, were prepared from the lyophilized preparation, kept in sealed ampoules in an ice-box.

^{203}Hg-labelled mercuric chloride (0.07 - 0.3 μCi/mg) was obtained from Instytut Badań Jądrowych (Świerk, Poland). For the assay of metallothionein a solution of 1 mg/ml ($0.5 - 1.0 \times 10^6$ c.p.m./ml) was used. Radioactivity of the samples was measured with scintillation counter USB.

Methods

Fresh liver or kidney was homogenized in a glass homogenizer in 0.9% NaCl solution (7 ml/1 g of tissue). To the two parallel samples containing 0.5 g of tissue, 0.4 ml of saline (sample *a*) or 0.4 ml (0.4 mg) of metallothionein (sample *b*) were alternatively added, followed routinely by 0.35 ml of $^{203}HgCl_2$ (350 μg Hg). The samples were mixed thoroughly and left for 30 min at room temperature, 1 ml of 10% trichloroacetic acid was then added and after 10 min the precipitate was centrifuged off at 3500 rev./min for 5 min. The supernatant was transferred to calibrated test tubes, the volume was adjusted to 5 ml with saline, and radioactivity was measured. As control, radioactivity of mercury added to 5 ml of saline was counted.

From the difference between samples (*b*) and (*a*) a calibration coefficient was calculated, which was further used for calculation of the amount of metallothionein in the sample (*a*). The results are expressed in milligrams of metallothionein per 1 gram of tissue.

In animals exposed to metals the content of metallothionein may reach values beyond the linearity of measurement. If the amount of mercury in the supernatant is over 2/3 of the amount bound by the supernatant with internal standard (0.4 mg), the tissue investigated has to be "diluted" with the homogenate from the same tissue of the control animal. (Generally, a five-fold "dilution" of the sample is

[1] Abbreviations used: TCA, trichloroacetic acid; HEDD, β-hydroxy-ethyl-1,2-dinitrophenyldisulphide.

satisfactory). In this case, liver and kidney from the metal-poisoned and control animals were homogenized separately in 0.9% NaCl (0.5 g of tissue/3.5 ml of saline) and mixed in 1:4 ratio. In this mixture metallothionein was assayed using the internal standard (0.4 mg) and 0.35 ml of $^{203}HgCl_2$ (350 μg Hg) in a total volume of 5 ml. $^{203}HgCl_2$ was added in the same amount to the homogenate (0.5 g of tissue) used for dilution.

For calculation of the results the following formula was used:

$$x = \frac{S(I_1 - 2I_2 m_2)}{m_1(I_3 - I_1)} \quad (1)$$

where x is the concentration of metallothionein expressed in mg/g of tissue;

m_1, the amount of tissue, in grams, taken for determination;

m_2, the amount of tissue, in grams, used for "dilution";

S, the amount of metallothionein, in mg, added as internal standard (the recommended amount is 0.4 mg);

I_1, the activity (c.p.m.) of the sample of the tissue studied ("diluted");

I_2, the activity (c.p.m.) of the tissue used for "dilution";

I_3, the activity (c.p.m.) of the sample ("diluted") with added internal standard of metallothionein.

Factor 2 appearing in formula (1) derives from the fact of constant amount of the tissues used in the assay: $m_1 + m_2 = 0.5$ g.

RESULTS AND DISCUSSION

So far, there was no method available for quantitative determination of metallothionein in animal tissues. Scanty information on variations of the level of this protein under the influence of cadmium administered *in vivo* has been obtained by spectrophotometric analysis of the eluates from gel chromatography: as observed by Wiśniewska-Knypl & Jabłońska (1970), metallothionein showed weak absorbance at 250 nm.

The method proposed in the present report, based on tracing of the protein with mercury, allows to determine metallothionein in a sufficiently broad range of concentrations. This enables the use of this assay in the studies on the effect of metals, administered *in vivo*, on the level of metallothionein.

Our previous studies showed no binding of Hg in kidney or liver with low-molecular compounds other than metallothionein (Jakubowski *et al.*, 1970; Piotrowski & Bolanowska, 1970). The analytical procedure has to include, however, separation of the Hg-metallothionein complex from the high-molecular proteins which complex with Hg. Of the procedures checked in this respect, the most efficient separations were achieved with the use of Rivanol (1%), sulphosalicylic acid (6%) and trichloroacetic acid (10%). The recovery of the metallothionein-mercury complex under these conditions was 80 - 62% and the respective metallothionein/total protein ratios in the

supernatant were 7.3 - 10.8 (total protein was determined according to Lowry *et al.*, 1951). The TCA-treatment, which resulted in the highest ratio, is recommended.

Figure 1 shows that the TCA-treated homogenate contained one major fraction of mercury compounds of molecular weight about 10 000, whereas the minor fraction of lower molecular weight contained but traces of mercury. The latter fraction has not been further studied.

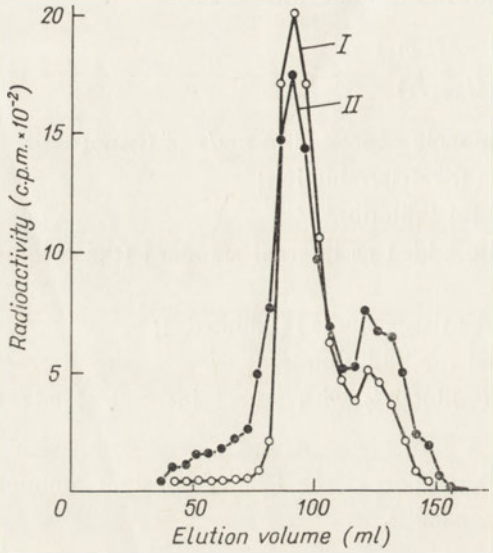

Fig. 1. Chromatogram of metallothionein (*I*) subjected to mercuric chloride (40 µg Hg/250 µg of metallothionein), and of the TCA-supernatant from the kidney homogenate (*II*), traced with mercuric chloride (350 µg Hg/0.5 g of tissue). Conditions: Sephadex G-75 column (1.9×45 cm), ammonium formate buffer, pH 8.1, ionic strength 0.1, flow rate about 1 ml/min; fractions of 5 ml were collected.

From Fig. 2 it may be seen that the content of mercury in the supernatant increased with the increasing concentrations of Hg in the homogenate, and maintained a plateau value over the range 500 - 1000 µg Hg/g of tissue. At concentrations exceeding the latter value, the Hg content in the supernatant rose rapidly indicating the presence of soluble, probably unchelated excess mercury. An analytical use of the mercury tracer may be made, therefore, only in the range of concentration 500 - 1000 µg Hg/g of tissue. Concentration of the same order of magnitude is also necessary to achieve a linear function of mercury content in supernatant *versus* metallothionein concentration (Fig. 3). Concentration of 700 µg Hg per gram of tissue was finally accepted in the method.

Incubation of the homogenate with mercury up to 22 h seems to be without essential effect on binding of Hg with metallothionein. In the homogenate samples which had been stored in the ice-box overnight, Hg binding was decreased probably due to decomposition of metallothionein in the homogenate *in vitro* (Table 1).

The calibration curves obtained from the homogenates of liver and kidney, with various amounts of added metallothionein, are presented in Fig. 4. It may be seen that the curves ran parallelly reflecting only difference due to the different content of metallothionein in the two tissues. An almost linear function was also obtained when the amount of Hg in the TCA-supernatant was related to the varying amount of the homogenate (Fig. 5).

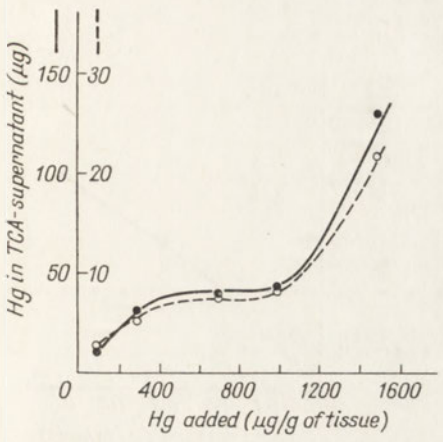

Fig. 2

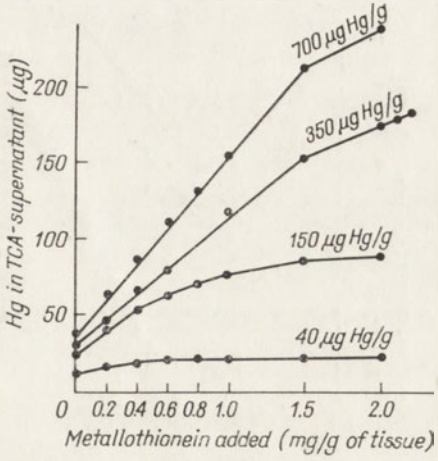

Fig. 3

Fig. 2. Effect of the mercury added to the homogenate of equine renal cortex on the level of mercury in the supernatant after deproteinization with trichloroacetic acid. The amount of homogenate corresponding to: ——, 0.1 g of tissue; - - -, 0.5 g of tissue, was used.

Fig. 3. Calibration curves for metallothionein with the indicated concentrations of mercury tracer (data calculated per 1 g of rat kidney tissue).

To evaluate the precision of the method proposed, a control experiment was performed on homogenate obtained from kidneys or livers. Ten parallel determinations with internal standards were performed with the same material. The obtained results (± S.E.) were: 0.77±0.034 mg/g for kidney and 0.26±0.0087 mg/g for liver. The precision of determinations were: ±4.4% for kidney and ±3.3% for liver.

From the data of Kägi & Vallee (1960) and of Pulido *et al.* (1966) it follows that the binding affinity of different metals for metallothionein increases in the order: zinc, cadmium, silver, mercury. From their data it is known that the native metallothionein may occur in animal and human kidneys as a complex with cadmium

Table 1

Effect of the incubation time on tracing of metallothionein in the kidney homogenate

A single dose of stable mercuric chloride (2 mg Hg/kg) was administered *in vivo* to rats. The homogenates were incubated with $^{203}HgCl_2$ and internal standard of metallothionein.

Homogenate stored for	Incubation with $^{203}HgCl_2$	Hg in the supernatant (μg)			
		no. of expts.	homogenate +^{203}Hg	no. of expts.	homogenate +metallothionein+ +^{203}Hg
<1 hr	1 - 5 min	2	21 - 22	2	63 - 64
	10 - 60 min	4	24 - 25	4	63 - 67
	22 h	1	27	1	68
22 h	30 min	1	17	5	55 - 58

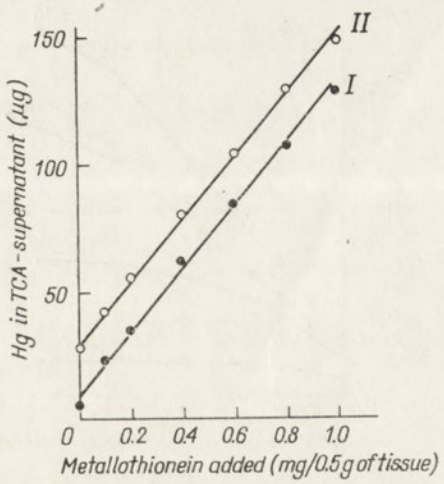

Fig. 4

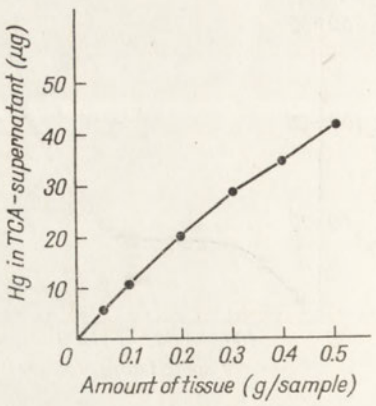

Fig. 5

Fig. 4. Calibration curves for metallothionein added to the homogenates of rat liver (*I*) and kidney (*II*). The amount of tissue used was 0.5 g.

Fig. 5. Content of mercury in the TCA-soluble fraction as a function of the amount of tissue (equine kidney cortex) taken. Both the volume of the samples and the ratio of added mercury tracer (700 μg Hg/g of tissue) were kept constant.

and zinc. Mercuric chloride used in an excess as a tracer substitutes cadmium and zinc in metallothionein complexes. Therefore, administration of high doses of cadmium to animals does not interfere with determination of metallothionein.

When metallothionein determinations are performed in the tissues of rats which have been exposed to mercury, it is obvious that the active mercury added *in vitro* for tracing of metallothionein will be diluted by stable mercury present in the tissue, and a tendency will arise to underestimate the level of this protein. However, this is corrected for by the use of internal standards of metallothionein, on the assumption that mercury undergoes efficient exchange.

Direct proof for this exchange is difficult to obtain since it requires repeated administration *in vivo* of Hg, which in turn leads to an increase in the levels of metallothionein due to induction of its biosynthesis (Piotrowski *et al.*, 1973). Therefore, the following experiments were performed: one group of rats received repeatedly stable mercury, another group ^{203}Hg of the same or higher activity than that applied for tracing metallothionein *in vitro*. Data given in Table 2 confirm the expected differences and their compensation by introducing internal standards, to give finally practically the same values for metallothionein content in both groups of experimental animals.

Preliminary results of determinations of metallothionein in rat tissues are presented in Table 3. Measurements were made in kidney and liver of the rats non-exposed and exposed for a long time to cadmium, which is known to induce biosynthesis of metallothionein.

Table 2

The effect of mercury administered in vivo *on the tracing of metallothionein by mercury* in vitro

Rat kidney homogenate was used as described in Methods; during the analytical procedure 350 μg of ^{203}Hg was added as tracer and 0.4 mg of metallothionein as internal standard.

Administration of Hg	Specific activity of tracer *in vitro*	Radioactivity (c.p.m./sample) following administration of active mercury			Level of metallothionein (mg/g of tissue)	Radioactivity (c.p.m./sample) following administration of stable mercury		Level of metallothionein (mg/g of tissue)
		Homogenate	Homogenate + tracer	Homogenate + internal standard + tracer		Homogenate + tracer	Homogenate + internal standard + tracer	
2.0 mg/kg, single dose (480 c.p.m./μg Hg)	480 c.p.m./μg Hg	7 676	21 900	41 340	0.88	14 940	31 770	0.70
		7 600	23 130	42 225	0.97	19 360	37 240	0.84
		6 080	16 670	37 600	0.67	14 370	32 060	0.66
0.5 mg/kg, 10 daily doses (1130 c.p.m./μg Hg)	200 c.p.m./μg Hg	56 774	46 260	63 814	2.1	28 400	36 800	2.8
		71 380	60 170	78 660	2.6	21 090	27 690	2.6
		71 000	61 100	79 730	2.6	23 640	30 960	2.3

Table 3

The level of metallothionein in the tissues of rats

Normal and cadmium-treated rats were used. Cadmium chloride was administered subcutaneously over a period of 8 months in a total dose of 40 mg/kg.

Tissue	Normal rats		Rats exposed to cadmium	
	Number of animals	Metallothionein (mg/g)	Number of animals	Metallothionein (mg/g)
Kidney	13	0.43 (0.26 - 0.56)	3	4.1 (3.0 - 5.6)
Liver	9	0.13 (0.07 - 0.30)	4	4.4 (2.8 - 9.0)

It may be concluded from the results presented that the procedure based on tracing of metallothionein with Hg is selective enough for evaluation of the content of this protein in animals exposed to heavy metals. The use of internal standards of appropriate purity and specificity is of great importance for the reliability of the method and validity of determinations of absolute amount of metallothionein in animal tissues.

One has to remember also that metallothionein from different animals may show essential differences. This reservation is, however, of minor importance in toxicological studies when relative changes in the level of metallothionein are determined in the same species.

The authors wish to thank Dr. Justyna M. Wiśniewska-Knypl for supplying rats subjected to long-term exposure to cadmium. Technical assistance of Mrs. Krystyna Kowalska and Mrs. Maria Walczak is highly appreciated. This research was carried out under the Polish-American Agreement 05-002-3, Occupational Health Program, U.S. Public Health Service.

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OZNACZANIE ZAWARTOŚCI METALOTIONEINY W TKANKACH ZWIERZĄT

Streszczenie

Opracowano metodę przybliżonego oznaczania zawartości metalotioneiny w wątrobie i nerkach szczurów. Metoda polega na kompleksowaniu metalotioneiny za pomocą ^{203}Hg i usunięciu wysokocząsteczkowych białek wiążących Hg za pomocą kwasu trójchlorooctowego. Ilość rtęci pozostałej w supernatancie jest proporcjonalna do zawartości metalotioneiny w tkance. W metodzie stosuje się wzorzec wewnętrzny metalotioneiny. Wstępne badania wykazały następujący poziom metalotioneiny w tkankach szczurów zdrowych: 0,1 mg/g (wątroba) i 0,4 mg/g (nerka); u szczurów, którym podano kadm, wartości te wzrosły do 4,4 mg/g w wątrobie i 4.1 mg/g w nerce.

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T. GOŁASZEWSKI, MARTA RYTEL and J. W. SZARKOWSKI

AN EXTRANUCLEAR DNA FRACTION METABOLICALLY MOBILIZED DURING THE EARLY STAGE OF RYE EMBRYO GERMINATION

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Extranuclear DNA from rye embryos germinated for 24 h and labelled *in vivo* with [^{3}H]thymidine, was fractionated on poly-L-lysine-kieselguhr column into two fractions. Only one of them, corresponding to about 25% of the total cell DNA, showed relatively high turnover.

The genome in dry plant seeds, or in isolated embryos, remains inactive, the replication and transcription processes being suppressed until water penetration is completed (cf. Chen & Osborne, 1970). Then the sequential triggering of macromolecule syntheses takes place (Jacob & Bovey, 1969; Chen & Osborne, 1970; Mory *et al.*, 1972).

So far, little is known on the metabolism of extranuclear DNA on the onset of germination. In the previous communication (Gołaszewski *et al.*, 1972) we have demonstrated that at the early stage of rye embryo germination, at the time when the metabolic activity of nuclear DNA is low, the pool of extranuclear DNA (bound with plastidial and mitochondrial fractions) is intensively labelled *in vivo* with [^{3}H] thymidine. The results of the present work indicate that during the first 24 h of rye embryo germination only a part of the extranuclear DNA fraction is actively metabolized.

MATERIALS AND METHODS

Special reagents. [6-^{3}H]Thymidine, sp. act. 28.50 Ci/mmol (I.R.P.U.R., Prague, Czechoslovakia); poly-L-lysine hydrobromide, type I, mol. wt. 150 000, orcinol (Sigma Chem. Co., St. Louis, Mo., U.S.A.); ribonuclease T_1 (crystalline) from *Aspergillus oryzae* (Sankyo Company Ltd., Tokyo, Japan); pronase from *Streptomyces griseus* (Koch.-Light Lab., Colnbrook, Bucks., England); cellulose, powdered (Whatman Biochemicals Ltd., Maidstone, Kent, England); kieselguhr, Hyfco-Super-Cel (Vulcascot, W. Germany). Other chemicals used were the same as in the previous work (Gołaszewski *et al.*, 1972).

Material. Rye (*Secale cereale* L.) embryos were purchased from Zakłady Zbożowo-Młynarskie (Świebodzin, Poland); the purity of the batches was 87%, humidity 13.4%.

Germination of embryos and labelling experiments were performed as described by Gołaszewski *et al.* (1972), except that for labelling 7.1 nmol of [6-^{3}H]thymidine (10 μCi/ml) was applied.

Isolation of extranuclear DNA. From the homogenate prepared as described previously (Gołaszewski *et al.*, 1972), the nuclear pellet was sedimented at 600 *g*, and from the supernatant plastids and mitochondria were sedimented at 12 000 *g* for 15 min. From this fraction DNA was isolated by the phenol method of Saito & Miura (1963) except that prolonged treatments of the DNA preparation with pancreatic ribonuclease (18 h at 37°C), then with ribonuclease T_1 (2 h at 37°C) and pronase (2 h at 37°C) were carried out. The adopted procedure has been described in more detail in the previous paper (Gołaszewski *et al.*, 1972).

*Fractionation of extranuclear DNA on the poly-*L*-lysine-kieselguhr (PLK) column.* The column was prepared according to Ayad & Blamire (1968). About 1.5 mg of DNA dissolved in 15 ml of 20 mM-phosphate buffer, pH 6.7, was applied to the PLK column and eluted with a stepwise NaCl concentration gradient (0.4 - 2.0 M) in the same buffer. The flow rate was 1 ml/min, and in 2 ml fractions DNA was estimated by measuring the extinction at 260 nm.

Estimation of DNA in subcellular fractions. From the homogenate prepared as described by Gołaszewski *et al.* (1972), three fractions: the nuclear (600 *g*, 3 min), plastidial (1000 *g*, 14 min) and mitochondrial one (12 000 *g*, 15 min) were isolated, and purified according to Nieman & Poulsen (1963). From the ether powder, RNA was extracted with 1 N-perchloric acid at 4°C and then DNA with 0.5 N-perchloric acid at 70°C for 15 min (2 - 3 consecutive extractions were needed) according to Ogur & Rosen (1950). In the extract, DNA was estimated spectrophotometrically according to Nieman & Poulsen (1963, 1967) at 265 nm and 310 nm.

Radioactivity determination. To 11 ml of the scintillation fluid (3 g of PPO and 0.3 g of POPOP per 1000 ml of toluene), 0.3 ml of DNA solution in water (about 200 μg) and 3.5 ml of cold absolute ethanol were added. Radioactivity was measured with an automatic liquid-scintillation spectrometer (Packard Tri-Carb, model 3003, efficiency for ^{3}H, 50%).

RESULTS AND DISCUSSION

The extranuclear DNA isolated from germinated rye embryos, gave on PLK column chromatography two distinct fractions (Fig. 1). The first one, comprising about 50% of the DNA recovered from the column, was eluted at 1.2 - 1.4 M-NaCl, and the second fraction at 2.0 M-NaCl concentration.

In the extranuclear DNA from rye embryos labelled *in vivo* with [^{3}H]thymidine, the radioactivity of the first fraction was low, whereas the second DNA fraction showed a rather high labelling (Table 1), its specific radioactivity being 17-fold higher than that of the first fraction. This indicates that at the early stage of rye

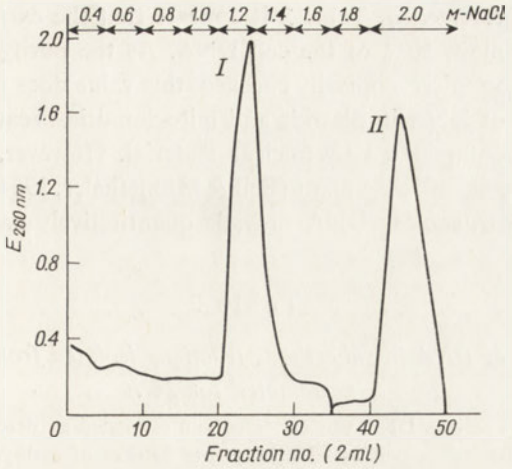

Fig. 1. Fractionation on poly-L-lysine-kieselguhr column of extranuclear DNA isolated from rye embryos germinated for 24 h. DNA, 1.5 mg in 15 ml of 20 mM-phosphate buffer, pH 6.7, was applied to the column equilibrated with the same buffer and eluted with a stepwise NaCl concentration gradient. In the collected fractions E_{260} was determined.

embryo germination only a part (about a half) of extranuclear DNA was metabolically active.

As in analogical labelling experiments with "dry" (ungerminated) embryos virtually no label was incorporated into extranuclear DNA, we can assume that during the first 24-h period of germination the initiation of extranuclear DNA metabolism occurs. Most probably, at that time the replication of the extranuclear DNA (bound with plastids and mitochondria) starts.

To learn what percentage of the total cell DNA is mobilized at the beginning of germination, we have determined the DNA distribution in subcellular fractions.

Table 1

Radioactivity of the extranuclear DNA fractions separated on the poly-L-lysine--kieselguhr column

Rye embryos germinated for 24 h were labelled *in vivo* for 15 min with [6-^{3}H]thymidine, and the extranuclear DNA was fractionated on the PLK column (see Fig. 1). For specific radioactivity determination the separated two fractions were dialysed against 20 mM-phosphate buffer, lyophilized to a small volume and DNA was estimated with diphenylamine reagent (Dische, 1955). The results of a typical experiment are given.

Fraction eluted with NaCl solution (M)		% of DNA eluted*	Specific activity (counts/min/mg of DNA)
I	1.2 - 1.4	47	1 126
II	2.0	53	19 217

* Calculated on the basis of E_{260} measurement.

From the results presented in Table 2 it appears that the extranuclear 1000 *g* and 12 000 *g* pellets contain 50% of the cell DNA. As the purity of the extranuclear fraction has not been microscopically checked, this value does not necessarily reflect the actual content of DNA in plastids and mitochondria because the 1000 *g* pellet could have been contaminated by nuclear material. However, it should be noted that in embryonic cells of some plant (Bell & Mühlethaler, 1964) and animal (David, 1966) organisms extranuclear DNA prevails quantitatively over nuclear DNA.

Table 2

Distribution of DNA in subcellular fractions isolated from rye embryos germinated for 24 h

From the subcellular fractions DNA was extracted and estimated as described in Methods. Mean values from three experiments performed on separate batches of embryos are given, with limit values in parentheses.

Subcellular fraction	Percentage of total cell DNA
600 *g* pellet	48 (42 - 52)
1 000 *g* pellet	34 (33 - 38)
12 000 *g* pellet	18 (15 - 20)

The results of the presented experiments indicate that at the onset of rye embryo germination an extranuclear DNA fraction, comprising not more than one-fourth of the total cell DNA, is metabolically mobilized. This DNA fraction, belonging to the extranuclear pool, may be involved in organellogenesis processes taking place during germination of plant embryos (cf. Nawa & Ashai, 1971).

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FRAKCJA POZAJĄDROWEGO DNA URUCHAMIANA METABOLICZNIE WE WCZESNYM OKRESIE KIEŁKOWANIA ZARODKÓW ŻYTA

Streszczenie

Znakowany *in vivo* [^{3}H]tymidyną pozajądrowy DNA zarodków żyta poddanych kiełkowaniu przez 24 godziny, rozdzielono na kolumnie wypełnionej poli-L-lizyną zmieszaną z ziemią okrzemkową na dwie frakcje. Tylko jedna z tych frakcji, obejmująca około 25% całkowitego DNA komórki, wykazuje względnie wysoką aktywność właściwą.

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ACID PHOSPHATASE III FROM POTATO TUBERS, MOLECULAR WEIGHT AND SUBUNIT STRUCTURE

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The molecular weight of the major form of acid phosphatase, acid phosphatase III, from potato tubers, calculated on the basis of the enzyme's behaviour in polyacrylamide gels of different concentrations, is about 96 000 daltons. The homogeneous form of the enzyme obtained by polyacrylamide gel - sucrose gradient electrophoresis gave on disc electrophoresis in the presence of sodium dodecyl sulphate a single fraction with a molecular weight of about 46 000 daltons. These results suggest that the potato acid phosphatase III is composed of two identical or nearly identical subunits.

Numerous non-specific acid phosphatases (EC 3.1.3.2) of animals, plants and microorganisms occur in multiple molecular forms, which have been isolated only in a few cases.

Acid phosphatase from potato has been studied mainly in the Laboratory of Lora-Tamayo (Andreu *et al.*, 1960; Alvarez, 1962; Lora-Tamayo *et al.*, 1969a,b) and by Hsu *et al.* (1966). The majority of these investigations concerned the kinetics of the enzyme but its more detailed molecular characteristics have not been described. Heterogeneity of potato acid phosphatase was reported by Kubicz & Morawiecka (1970, 1971) who demonstrated that on electrophoresis in polyacrylamide gel the enzyme appeared in three molecular forms.

The aim of the present work was to obtain a homogeneous preparation of the predominant form of the enzyme, acid phosphatase III, and to determine the molecular weight of the enzyme and its subunits.

MATERIAL AND METHODS

Chemicals. Acrylamide, *N,N'*-methylenebisacrylamide, 2-mercaptoethanol, sodium 1-naphthyl phosphate, sodium *p*-nitrophenylphosphate, trypsin 1×cryst. (Koch-Light, Colnbrook, Bucks., England), sodium phenylphosphate, bovine serum albumin (B.D.H. Ltd, Poole, Dorset, England); *N,N,N',N'*-tetramethylethylenediamine (Eastman Organic Co., U.S.A.); pepsin 3×cryst. (Nutritional Biochem. Corp.,

Cleveland, Ohio, U.S.A.); lactate dehydrogenase from rabbit muscles (Boehringer, Mannheim, G.F.R.); Sephadex G-75 (Pharmacia, Uppsala, Sweden); Fast Blue B (George T. Gurr Ltd, London, England); Amido Black 10 B (Merck, Darmstadt, G.F.R.); Coomassie Brilliant Blue, R-250 (Colab. Lab., Chicago, U.S.A.); tris (Fluka AG, Buchs SG, Switzerland); sodium dodecyl sulphate (Schuchardt, München, G.F.R.). Human haptoglobin type 1-1 was a gift from Prof. Dr. Wanda Dobryszycka.

Purification of potato acid phosphatase on Sephadex G-75. The starting material was a lyophilized crude preparation obtained from potato extract according to Mejbaum-Katzenellenbogen & Morawiecka (1959). A sample, about 480 mg of protein (spec. act. 0.4 unit/mg) was dissolved in 10 ml of 0.02 M-acetate buffer, pH 5.0, applied to the Sephadex G-75 column (2.5×45 cm) and eluted with the same buffer (Fig. 1). The most active fractions (no. 6 - 16) containing 25% of the applied protein were pooled. The specific activity of this preparation was 0.8 unit/mg, and the total activity, 97 units. The preparation was concentrated to about 20 ml, applied again to the same Sephadex G-75 column and eluted as above. The pooled active eluates contained 62 mg of protein of specific activity 2.38 unit/mg, which corresponds to 3.5-fold purification of the starting material. This purified preparation was lyophilized and used for the experiments.

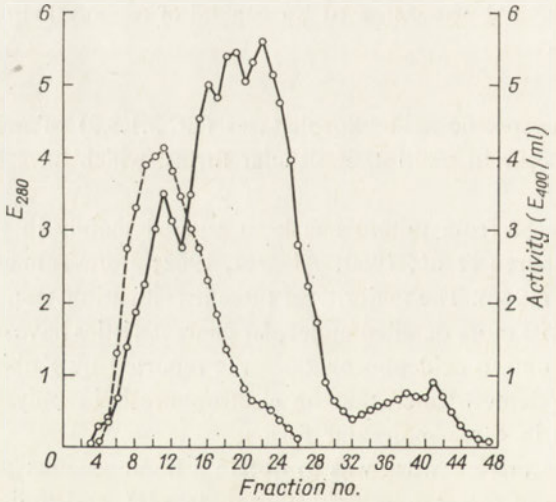


Fig. 1. Purification of crude potato acid phosphatase on Sephadex G-75. About 480 mg of protein was dissolved in 10 ml of 0.02 M-acetate buffer, pH 5.0, and applied to the column (2.5×45 cm) which was equilibrated and eluted with the same buffer. The void volume (about 62 ml) was discarded, then fractions of 4 ml were collected.——, E_{280}; - - -, activity, measured as the amount of liberated *p*-nitrophenol.

Acid phosphatase activity determination. The appropriate amount of enzyme in 1.4 ml of 0.1 M-acetate buffer, pH 5.0, and 0.5 ml of 0.01 M-sodium phenylphosphate was incubated for 30 min at 37°C. The reaction was stopped by adding 2 ml of cold 5% trichloroacetic acid, and the liberated orthophosphate was determined in the

whole sample by the method of Fiske & Subbarow. One unit was defined as the amount of enzyme required to liberate 1 μmol of inorganic phosphate per minute under these conditions. The elution of phosphatase from the Sephadex G-75 column was followed as described by Verjée (1969) with *p*-nitrophenylphosphate as substrate.

Protein was determined by the turbidimetric tannin method according to Mejbaum-Katzenellenbogen (1955).

Polyacrylamide gel electrophoresis was performed on 7.5% gels (0.5×6.5 cm) at pH 9.5 according to Ornstein (1964) and Davis (1964) or at pH 4.3 according to Reisfeld *et al.* (1962). When electrophoresis was carried out at pH 9.5, the lower gel contained 0.0005% of riboflavin and the upper one, 20% of sucrose. After separation, the protein was stained with 1% Amido Black 10 B in 7% acetic acid and the activity of acid phosphatase was located with 1-naphthyl phosphate and Fast Blue B (5 mg of each per 5 ml of 0.2 M-acetate buffer, pH 5.0).

Molecular weight determination. For native phosphatase III, electrophoresis in polyacrylamide gels of different concentrations according to Hedrick & Smith (1968) was applied. Molecular weight of the subunits was determined by disc electrophoresis on 10% polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS) at pH 7.2 by the method of Shapiro *et al.* (1967) as described by Weber & Osborn (1969) and Dunker & Rueckert (1969).

The following standard markers were used: bovine serum albumin (monomer, dimer, trimer), mol.wt. of monomer 66 000 (Dunker & Rueckert, 1969), pepsin, mol. wt. 35 000 (Bovey & Yanari, 1960), trypsin, mol. wt. 23 000 (Desnuelle, 1960), lactate dehydrogenase from rabbit muscle, mol. wt. 135 000 (Schwert & Winer, 1963), and human haptoglobin type 1-1, mol. wt. 89 000 (cf. Zwaan, 1967).

RESULTS

On disc electrophoresis at pH 9.3 the enzyme preparation purified on Sephadex G-75, similarly as the crude starting material (Fig. 2A,B), gave three active fractions designated $AcPh_1$, $AcPh_2$ and $AcPh_3$. A similar heterogeneity of potato acid phosphatase was observed also at pH 4.3 (Fig. 2C).

The molecular weight of the dominant form of the enzyme, $AcPh_3$ (acid phosphatase III) was determined according to Hedrick & Smith (1968) using gels of different concentrations. The slope of logarithm of relative mobility (R_m) *versus* gel concentration was determined for standard proteins and phosphatase III; the molecular weight of the enzyme obtained from this plot (Fig. 3) in three independent experiments was 92 000, 98 000 and 98 000.

The plot of log R_m *versus* gel concentration for the three molecular forms of the enzyme gave parallel lines, which suggests that these forms do not differ in molecular weight. The buffer system of Hedrick & Smith (1968) is, however, limited to proteins with isoelectric point lower than pH 6.0 and the slowest migrating $AcPh_1$ showed in this system very low electrophoretic mobility. When this buffer system was replaced by that of Davis (1964) and Ornstein (1964) of pH 9.5, all three acid phosphatases

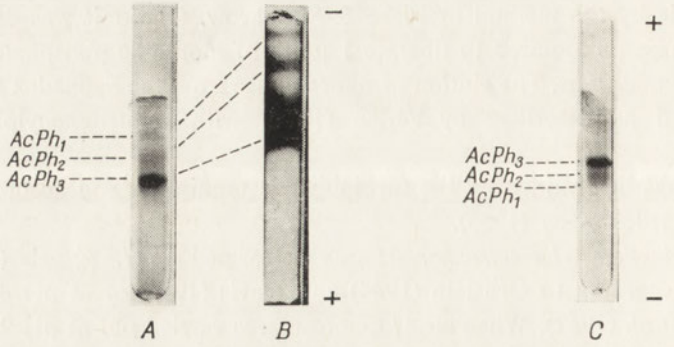


Fig. 2. Zymograms of *A*, crude and *B*, purified preparation of potato acid phosphatase. Electrophoresis in 7.5% polyacrylamide gels at pH 9.5 was performed according to Ornstein (1964) and Davis (1964). *C*, The purified preparation separated by electrophoresis at pH 4.3 according to Reisfeld *et al.* (1962). Enzymic activity was located using 1-naphthol phosphate and Fast Blue B at pH 5.0.

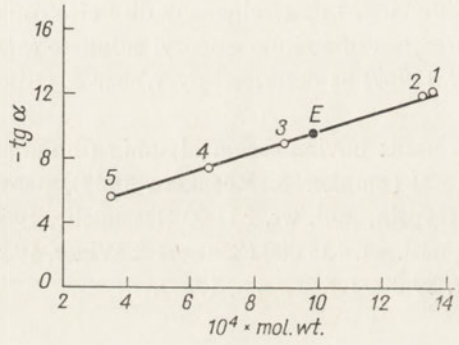


Fig. 3

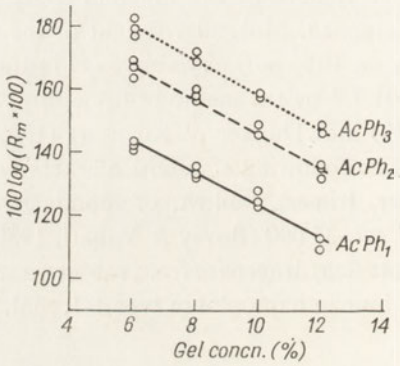


Fig. 4

Fig. 3. Molecular weight determination of acid phosphatase III by disc electrophoresis at pH 8.5 according to Hedrick & Smith (1968). The values of -tg *a* were calculated from the plots of log R_m *versus* gel concentration. *E*, Acid phosphatase III; standard proteins: *1*, lactate dehydrogenase; *2*, dimer of bovine serum albumin; *3*, haptoglobin type 1-1; *4*, monomer of bovine serum albumin; *5*, pepsin. For details see Materials and Methods.

Fig. 4. Effect of different gel concentrations on the mobility of three forms of acid phosphatase from potato. Electrophoresis was performed at pH 9.5 in the buffer system of Davis (1964) and Ornstein (1964). The Bis/acrylamide monomer weight ratio was 1:30 according to Hedrick & Smith (1968). For details see Materials and Methods.

were in anionic form. The obtained parallel lines (Fig. 4) indicate that the three forms of the enzyme had the same molecular weight.

The homogeneous preparation of acid phosphatase III was obtained by polyacrylamide gel - sucrose density gradient electrophoresis according to Shuster & Schrier (1967). A sample, 10 mg of protein, was dissolved in 5 ml of the electrolyte buffer containing 10% of sucrose. After 6 h of electrophoresis, from the cathodic arm of the U-tube 1 ml fractions were collected and protein determined (Fig. 5). Fractions 3 - 5, 8 - 11 and 12 - 14 were combined, dialysed for 48 h against 0.01M-acetate

buffer, pH 5.0, and then analysed by disc electrophoresis. In fractions no. 12 - 14 homogeneous acid phosphatase III was obtained, which appeared as a single band both on the zymogram and proteinogram (photographs in Fig. 5).

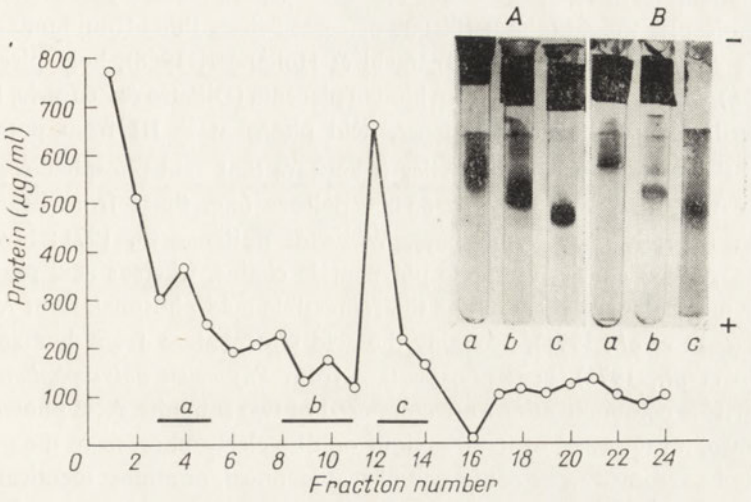
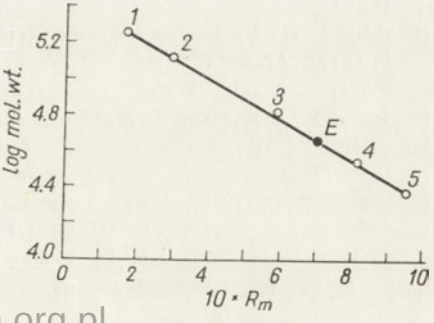


Fig. 5. Isolation of homogeneous acid phosphatase III by polyacrylamide gel - sucrose gradient electrophoresis. About 10 mg of protein dissolved in 5 ml of the electrolyte buffer containing 10% of sucrose and bromophenol blue, was subjected to electrophoresis at 500 V. The electrophoresis was terminated when the dye had migrated 4.5 cm into the sucrose gradient (about 6 h). The fractions *a*, no. 3 - 5; *b*, 8 - 11; *c*, 12 - 14, were pooled and submitted to disc gel electrophoresis. The photographs at the upper right represent: *A*, proteinograms (about 80 μg of protein) and *B*, zymograms (about 120 μg of protein) of the respective fractions.

The obtained homogeneous preparation and standard proteins were incubated with 1% SDS, 4 M-urea and 1% 2-mercaptoethanol for 60 min at 45°C, then the samples were subjected to disc electrophoresis in the presence of SDS (Fig. 6). Acid phosphatase III treated with the denaturing agents gave one protein fraction with a mobility intermediate between that of albumin monomer and pepsin. Its molecular weight in several experiments ranged from about 44 000 to 46 000 daltons. These results suggest that the acid phosphatase III of about 96 000 mol. wt. is composed of two identical, or almost identical, subunits.

Fig. 6. Polyacrylamide gel electrophoresis in the presence of SDS, of homogeneous acid phosphatase III. Standard proteins and the enzyme were incubated in the presence of 1% SDS, 4 M-urea and 1% 2-mercaptoethanol for 60 min at 45°C. Samples of 10 - 20 μg of protein were submitted to electrophoresis in 10% gel, at room temperature, at 7 - 9 mA per tube (Dunker & Rueckert, 1969). Proteins were stained with Coomassie Brilliant Blue. *E*, Acid phosphatase III; *1*, *2*, *3*, trimer, dimer and monomer, respectively, of bovine serum albumin; *4*, pepsin; *5*, trypsin.



DISCUSSION

The molecular weight of acid phosphatases obtained from various sources differs widely, ranging from 13 000 to about 100 000 daltons. To acid phosphatases of the highest molecular weight (about 100 000 daltons) belong those from human prostate (Derechin *et al.*, 1971), rat liver (Igarashi & Holländer, 1968), hog spleen (Chersi *et al.*, 1966), acid phosphatase II from human placenta (DiPetro & Zengerle, 1967) and, as demonstrated in the present paper, acid phosphatase III from potato tuber.

From the so far performed studies it appears that acid phosphatases of lower molecular weight, from 13 000 to 55 000 daltons (e.g. those from bean sprouts, Felenbok, 1970; human erythrocytes, White & Butterworth, 1971; bovine liver, Heinrikson, 1969) consist of single polypeptide chains, whereas acid phosphatases of higher molecular weight, such as the repressible acid phosphatase of *Neurospora crassa* (Jacobs *et al.*, 1971), probably the acid phosphatase from human prostate (Derechin *et al.*, 1971), acid phosphatase from *Physarum polycephalum* (Dr. M. Wolny, private communication), are composed of two subunits. Acid phosphatase III from potato, on the basis of its behaviour on disc electrophoresis in the presence of SDS, may be assumed to be composed of two identical, or almost identical, subunits of molecular weight about 46 000 daltons. The three molecular forms of acid phosphatase from potato tubers have the same molecular weight, therefore it seems that they all have a dimeric structure.

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KWAŚNA FOSFATAZA ZIEMNIAKA — CIĘŻAR CZĄSTECZKOWY I STRUKTURA PODJEDNOSTKOWA

Streszczenie

Ciężar cząsteczkowy głównej formy molekularnej kwaśnej fosfatazy ziemniaka, fosfatazy III, oznaczony w elektroforezie dyskowej w żelach o różnym stężeniu wynosi około 96 000 daltonów. Homogenną formę tego enzymu otrzymano za pomocą rozdziału w żelu poliakryloamidowym z gradientem sacharozy. W elektroforezie dyskowej w obecności siarczanu dodecylu homogenny enzym występuje jako pojedyncza frakcja o ciężarze około 46 000 daltonów. Wyniki te wskazują, że kwaśna fosfataza III ziemniaka jest zbudowana z dwóch identycznych lub prawie identycznych podjednostek.

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METABOLISM OF A NEW SERIES OF OLEANOLIC ACID GLYCOSIDES IN *CALENDULA OFFICINALIS* SHOOTS

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The dynamics of labelling of the glycosides derived from oleanolic acid 3-glucoside were investigated in the shoots of *Calendula officinalis*. It was shown that galactose moieties are added one by one in position 4′ of oleanolic acid 3-glucoside but glucose moieties are presumably transferred in transglycosylation reaction from unknown donors as two- or three-saccharide chains to position 3′ of these glycosides.

A number of new triterpenic glycosides, derivatives of 3-glucoside or 3-glucuronoside of oleanolic acid, have been isolated from *Calendula officinalis* (Kasprzyk & Wojciechowski, 1967; Wojciechowski *et al.*, 1971). The results of colorimetric determinations have shown large differences in distribution of both series of glycosides in various parts of *C. officinalis* plants during vegetation. In the shoots and roots of young plants and in the flowers only glucuronosides are present. Glucosides appear first in the shoots of 30-day-old plants and then in the roots at the 60th day of vegetation (Miller, 1970; Wasilewska, 1970). The level of glycosides of both series in the shoots, as well as the level of glucuronosides in the roots, increases continuously up to the flowering stage and then decreases, but the level of glucosides in the roots increases till the end of vegetation.

The sequence of reactions in biosynthesis of the oleanolic acid glucuronosides was studied by estimating dynamics of labelling of individual glycosides of this series. In these experiments [1-^{14}C]acetate was used as the precursor. It has been shown (Kasprzyk *et al.*, 1970) that five glucuronosides differing in the number of sugar components are formed as a result of a stepwise addition of single sugar moieties.

The present paper deals with biosynthetic sequences of the second series of these triterpenic glycosides, i.e. the oleanolic acid 3-glucosides.

MATERIAL AND METHODS

Material. Calendula officinalis plants var. Radio were cultivated in phytothrone under conditions described previously (Kasprzyk *et al.*, 1968).

Administration of $^{14}CO_2$. One-month-old shoots of *C. officinalis* were administered with 100 μCi of $^{14}CO_2$ (spec. act. 13.5 mCi/mmol) per gram of fresh weight

during one hour at light intensity of 40 000 lux as described by Kasprzyk *et al.* (1971). The plants were then transferred into vessels with tap water and illuminated for 8 hours per day with 3000 lux. The plants were processed after 1, 5, 15, 25, 50, 200, 260 and 400 h.

Fractionation of the material. The excised shoots and roots were cut to small pieces and extracted 5 times with boiling methanol. An equal volume of water was added to the extract, methanol was distilled off and the aqueous residue was subsequently extracted 4 times with butanol.

Preparative chromatography. Glycosides present in the butanolic extract were separated on thin-layer chromatograms with silica gel (Kieselgel G Merck) in the chloroform - methanol - water system (63 : 32 : 5, by vol.). This system was used for separation of glucosides I, II, III and IV, the glucosides F and VII being separated after elution, by means of thin-layer chromatography with silica gel in ethyl acetate - acetic acid - water (3 : 1 : 3, by vol.).

Acid hydrolysis of glycosides. Individual glycosides were subjected to acid hydrolysis as described by Kasprzyk *et al.* (1970). The released oleanolic acid was separated using plates with silica gel in chloroform - methanol (95 : 5, v/v) and localized by comparing with a standard after visualization with 50% H_2SO_4. The band of radioactive oleanolic acid was identified by autoradiography.

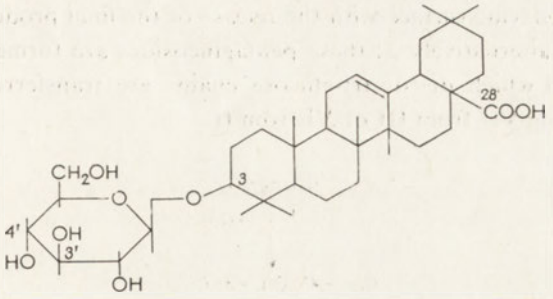
Radioactivity measurements. Radioactive oleanolic acid was eluted from silica gel with ethyl ether, and radioactivity was counted in the scintillation counter as described previously (Strüby *et al.*, 1972).

Quantitative determination of oleanolic acid. This was carried out using the colorimetric method with $CoCl_2$, described by Fonberg & Kasprzyk (1965). Standard curve was prepared with the compounds tested. Measurements were performed by means of Carl Zeiss VSU-1 spectrophotometer.

RESULTS AND DISCUSSION

The profile of changes in radioactivity of total oleanolic acid bound in glucuronosides and glucosides was investigated in plants from 1 to 260 h after $^{14}CO_2$ supply. It has been shown that labelling of oleanolic acid in glucuronosides was at the same level as labelling with [1-^{14}C]acetate and increased continuously during 260 h. On the contrary, labelling of oleanolic acid bound in 3-glucosides was severalfold higher and exhibited maximum after 50 h. The structure of 3-glucosides of oleanolic acid are given in Scheme 1.

Besides monoglucoside I, diglucoside II and pentaglucosides VI and VII, which were detected in the shoots of *C. officinalis* by colorimetric assay, also triglucoside III was labelled when $^{14}CO_2$ was used as the precursor. We could not find radioactivity in tetraglucosides IV and V, which were detected only in the old roots of *Calendula*, where presumably these glucosides are formed as the result of degradation of pentaglucosides transported to this organ from the shoots. The labelling pattern of particular glucosides is illustrated in Fig. 1. After 1 h, labelling of monoglucoside I



I 3-glucoside
II 3-(4′-galactosyl)-glucoside
III 3-(4′-digalactosyl)-glucoside
IV 3-(3′-glucosyl,4′-galactosyl)-glucoside
V 3-(3′-diglucosyl,4′-galactosyl)-glucoside
VI 3-(3′-diglucosyl,4′-digalactosyl)-glucoside
VII 3-(3′-triglucosyl,4′-galactosyl)-glucoside
VIII 3-(3′-diglucosyl,4′-galactosyl)-glucoside,28-glucoside

Scheme 1. Structure of oleanolic acid glucosides isolated from *Calendula officinalis* L.

was already declining. For II the maximum of ^{14}C incorporation was demonstrated between 15 and 25 h, for III at 25 h, and for pentaglucosides VI and VII, at 50 h. After reaching the maximum, radioactivity of all glucosides decreased rather sharply, suggesting their further transformations. These results indicate the following course of transformations (illustrated in Scheme 2); glucoside I is formed first and it is the precursor of all other glucosides of this series. Addition of one molecule of galactose in position 4′ of glucose in I results in formation of II. At this point, metabolic pathway forks leading on one hand to glucoside VI, and on the other to VII. Glucoside III is formed due to the addition of a second galactose molecule to the glucoside II. Glucose molecules can be added in two ways during biosynthesis of II, VII and VI; 1, in a non-stop reaction individual glucose molecules are transferred to II or

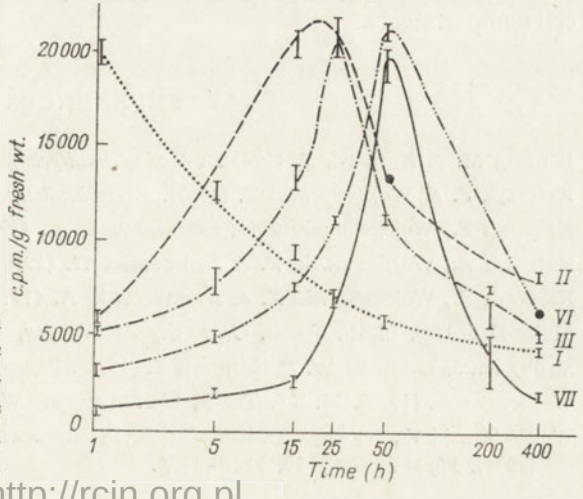
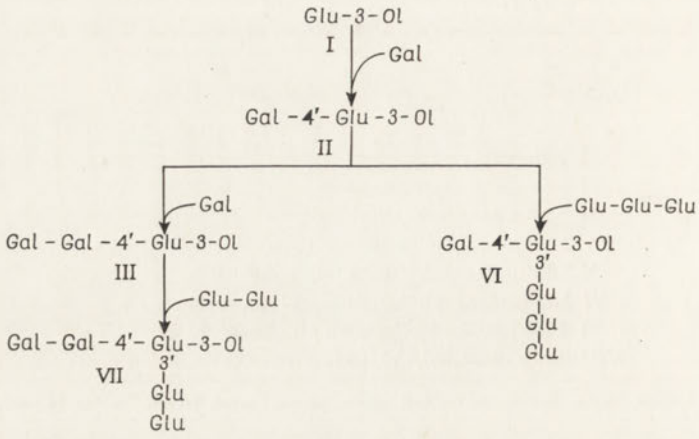


Fig. 1. The dynamics of labelling with $^{14}CO_2$ of the 3-glucosides of oleanolic acid in the shoots of *Calendula officinalis*. Figures in the diagram refer to the particular 3-glucosides (cf. Scheme 1). Vertical bars correspond to limit values.

III on the same enzyme surface with the release of the final products — pentaglucosides VI or VII; alternatively 2: these pentaglucosides are formed in transglycosylation reaction in which di- or tri-glucose chains are transferred from unknown precursors yielding VII from III or VI from II.



Scheme 2. Transformation of the glucosides derived from 3-glucoside of oleanolic acid in shoots of *Calendula officinalis*.

The results obtained indicate that in *C. officinalis* shoots different routes of biosynthesis of glycosides of both series could be operating. Derivatives of oleanolic acid 3-glucoside are rapidly formed in the shoots of four-week-old plants as concluded from the very high rate of radioactivity incorporation into these compounds. In the same period, biosynthesis of 3-glucuronosides proceeds much slower as indicated by a slow, but continuous, increase of radioactivity incorporated into oleanolic acid bound in these compounds.

It seems from the presented data that there is no metabolic interlink between these two series of glycosides — possibly as a result of their localization in different cell compartments.

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METABOLIZM NOWEJ SERII GLIKOZYDÓW KWASU OLEANOLOWEGO W PĘDACH *CALENDULA OFFICINALIS* L.

Streszczenie

Badano włączanie piętna z $^{14}CO_2$ w glukozydy — pochodne 3-glukozydu kwasu oleanolowego — w pędach *Calendula officinalis*. Wykazano, że cząsteczki galaktozy są dołączane kolejno do 3-glukozydu kwasu oleanolowego w pozycję 4′, natomiast cząsteczki glukozy prawdopodobnie są dołączane jako łańcuchy dwu - lub trójsacharydowe.

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SYNTHESIS OF XANTHOSINE-CONTAINING DINUCLEOSIDES BY RNase T_1

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1. It has been shown that guanylic acid-specific ribonuclease (EC 2.7.7.26) catalyses the synthesis of dinucleoside monophosphates of the general type XN, using xanthosine cyclic phosphate and several pyrimidine nucleosides as substrates. Conditions for optimal yield were established. The reaction products were resolved by paper and column chromatography and their identity established on the basis of spectrophotometric and chromatographic data. 2. The enzymically synthesized phosphodiester bond was shown to be 3′-5′. 3. The yields were fairly high, up to 45% relative to starting xanthosine cyclic phosphate. 4. All substrates and products can be readily recovered by chromatography, and repeatedly used. 5. The procedure can be applied to large-scale preparations of dinucleoside monophosphates with xanthosine at the 5′ end; those should prove valuable in conformational studies on oligonucleotides, and in applications to studies of polynucleotides structures.

The search for simple syntheses of oligonucleotides with known sequence is of interest in investigations of the forces stabilizing the structure and conformation of natural and synthetic nucleic acids. Knowledge of these forces facilitates elucidation of the nature of interactions between nucleic acids in model systems and *in vivo*.

RNase T_1[1] (EC 2.7.7.26) is known to be an enzyme splitting phosphodiester bonds adjacent to guanylate and xanthylate residues, first forming oligonucleotides which terminate in guanosine or xanthosine 2′,3′-cyclic phosphates and then hydro-

[1] The abbreviations used were those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [*Eur. J. Biochem.* **15**, 203 (1970)]: aC, 1-β-D-arabinofuranosylcytosine; Cm, 2′-*O*-methylcytidine; m_2^3′Cm, 2′,3′-*O*-dimethylcytidine; m^3′C, 3′-*O*-methylcytidine; m^6C, 6-methylcytidine; G2′(3′)p; mixture of 2′ and 3′ phosphates of guanosine, with similar connotation for other nucleotides; X>p, 2′,3′-cyclic phosphate of xanthosine; UU, uridylyl(3′-5′)uridine dinucleoside monophosphate; CC, cytidylyl(3′-5′)cytidine dinucleoside monophosphate; XN, general formula for dinucleoside monophosphate with xanthosine at the 5′ end, and other nucleoside at the 3′ end; XNp, dinucleoside diphosphate with terminal 2′(3′)phosphate; poly(X), polyriboxanthylic acid; poly(U), polyribouridylic acid; poly(A), polyriboadenylic acid; poly(XU), random copolymer of X and U; RNase T_1, guanylic acid-specific ribonuclease.

lysing these to guanosine and xanthosine 3′-phosphate termini (Egami *et al.*, 1964). It has been shown that the former reaction can be reversible, and the formation of oligoguanylic acids and oligonucleotides with guanosine at the 5′ end with RNase T_1 has been frequently reported (Sato-Asano & Egami, 1958; Hayashi & Egami, 1963; Sekiya *et al.*, 1968; Podder & Tinoco, 1969; Mohr & Thach, 1969; Rowe & Smith, 1971).

Xanthylic acid-containing polymers and oligomers are substrates for RNase T_1 (Whitfeld & Witzel, 1963; Egami *et al.*, 1964; Fikus & Shugar, 1969; Tichy & Fikus, 1970). Therefore it appeared useful to examine the synthetic activity of RNase T_1 towards xanthosine 2′,3′-cyclic phosphate as one of the substrates and various nucleosides as the second component of the reaction.

MATERIALS AND METHODS

Commercial RNase T_1 (Sankyo Co. Ltd, Tokyo, Japan) was used. Its activity was occasionally checked by the procedure of Egami *et al.* (1964). RNase A was purchased from Sigma Chem. Co. (St. Louis, Mo., U.S.A.). Bacterial alkaline phosphatase was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). RNA (Austranal) was fractionated on a Sephadex G-25 column, and the long-chain fractions were used for enzymic activity measurements. Guanosine 2′(3′)phosphate was purchased from "Waldhof-Aschaffenburg" A.G. (Mannheim, G.F.R.). Xanthosine 2′(3′)phosphate was obtained by deamination of guanosine 2′(3′)phosphate, according to the procedure given for guanosine by Shapiro & Pohl (1968). The experimental conditions were chosen so as to reduce the formation of side products [e.g. 2-nitroinosinic acid, Shapiro (1964)], by maintaining the temperature at 50°C, pH 5.1 and the concentration of sodium nitrite constant by continuous addition during incubation. Deamination was followed by measurements of the UV spectra of the properly diluted reaction mixture (cf. Shapiro & Pohl, 1968) and rechecked by chromatography on DEAE-cellulose paper after desalting on active charcoal. From the time of attaining, according to both control methods, a constant level of xanthosine 2′(3′)phosphate the reaction was continued for another 20 h. Incubation was stopped after 44 - 48 h by precipitation of the barium salt of xanthosine phosphate with twice the volume of ethyl alcohol. Thus, the inorganic salts remained in solution and xanthosine phosphate was separated by centrifugation after several hours' refrigeration. The precipitate was washed with 96% ethanol and acetone, and dried under vacuum. The yield was about 80%. No traces of contamination were detected by paper chromatography. This preparation was used for the cyclic phosphate synthesis without further purification, after conversion of the barium salt of xanthosine phosphate to the free acid on Dowex 1X2 (H^+).

Xanthosine 2′,3′-cyclic phosphate (X>p) was prepared according to Shugar (1967), by adaptation of his procedure for preparation of uridine 2′,3′-cyclic phosphate. Xanthosine cyclic phosphate was kept at -30°C under acetone and withdrawn for the enzymic reaction just prior to use. Cytidine cyclic phosphate was obtained as described by Shugar (1967). Uridine and cytidine were commercial preparations

(Waldhoff-Aschaffenburg A.G.). 6-Methylcytidine was a gift of Dr. M. Świerkowski; 1-β-D-arabinofuranosylcytosine, 2′-*O*-methyl-, 3′-*O*-methyl- and 2′,3′-dimethyl-cytidines were kindly offered by Mr. T. J. Kuśmierek. DEAE-cellulose (Whatman DE 11) was used as bicarbonate for column chromatography. Dimethylformamide and triethylamine were freshly distilled prior to use.

For TLC, cellulose-F plates (E. Merck, AG Darmstadt, G.F.R.) were used. Ascending paper chromatography was run on Whatman 1, 3*MM* and Whatman DE 81 papers. The following solvents were used:

A. Ethanol - 1 M-ammonium acetate, pH 7.5 (7 : 3, by vol.).

B. Saturated ammonium sulphate - 1 M-ammonium acetate - isopropanol (80 : 18 : 2, by vol.).

C. Propanol - ammonia - water (5.5 : 10 : 3.5, by vol.).

Molar coefficients for 6-methyl cytidine communicated by Dr. M. Świerkowski were as follows: λ_{max} (pH 2): 278 nm ($\varepsilon = 14.7 \times 10^3$); (pH 7, 12): 271 nm ($\varepsilon = 9.5 \times 10^3$). Molar coefficients for cytidines methylated on the ribose moiety were taken from Kuśmierek *et al.* (1973), for 1-β-D-arabinofuranosylcytosine from Kikugawa & Ichino (1972), for xanthosine from Cavalieri *et al.* (1954), and for uridine and cytidine from Calbiochem Tables.

The yield of the dinucleoside synthesis was expressed as percentage of xanthosine cyclic phosphate incorporated into dinucleoside. The UV spectra were recorded on a MOM-202 spectrophotometer (Hungary); pH measurements were taken on pH-meter type 28 (Radiometer, Copenhagen).

RESULTS AND DISCUSSION

RNase T_1 *action: conditions, type of the synthesized phosphodiester bond*

For the synthesis of xanthosine-containing dinucleosides, conditions were selected so as to avoid self-condensation of the cyclic phosphate (Mohr & Thach, 1969). The dinucleoside monophosphates were detected by chromatography and eluted for further identification (Table 1). The identification of dinucleoside phosphate was performed by alkaline hydrolysis (0.3 M-KOH, 37°C, 18 h) and subsequent paper chromatography of the products of hydrolysis. They were identified by their R_F values and, after elution, estimated spectrally. Comparison was made of the additive spectra of xanthosine and of a given nucleoside with that of the presumed dinucleoside monophosphate.

It might be of interest to mention that poly(X) and dinucleosides phosphates of the general type XN proved to be resistant to the action of snake venom phosphodiesterase under conditions in which poly(U) or poly(A) are readily hydrolysed to monomers. Random copolymers, viz. poly(XU), showed partial susceptibility to snake venom phosphodiesterase and yielded uridylic acid and a mixture of oligonucleotides.

Dinucleosides of the type XN, obtained with the use of RNase T_1, were relatively stable to hydrolysis by this enzyme (Whitfeld & Witzel, 1963). Consequently, the

Table 1

R_F *values of substrates, and of dinucleoside phosphates synthesized with RNase* T_1, *on chromatography in solvent* A

Nucleoside		Nucleotide		Respective dinucleoside phosphate	
C	0.65	X2′(3′)p	0.21	XC	0.30
U	0.72	X>p	0.38	XU	0.33
m^6C	0.80			Xm^6C	0.27
Cm	0.80			XCm	0.34
$m^{3'}C$	0.80			$Xm^{3'}C$	0.34
$m^{3'}_2Cm$	0.82			$Xm^{3'}_2Cm$	0.45
aC	0.76			XaC	0.30

synthesis was rather efficient (yield up to 50%) even after prolonged incubation periods (up to 100 h). The solubility of the nucleoside component is an obvious limitation of the method. No traces of xanthosine phosphate were detected after 40 - 60 h of incubation under our experimental conditions. Incubation for 120 h without enzyme led to spontaneous hydrolysis of xanthosine cyclic phosphate to xanthosine 2′(3′)phosphate (14%). Concentrations of both substrates were as follows: 0.1 - 0.8 M for the nucleoside and two orders of magnitude lower for xanthosine cyclic phosphate (as specified below). In order to compensate for dilution due to addition of the components, the resulting incubation mixture (saturated nucleoside solution plus xanthosine cyclic phosphate solution plus buffer) was, prior to addition of the enzyme, concentrated at room temperature in a stream of cold air to roughly 1/3 of its volume. The starting concentrations of both substrates were determined by chromatography.

In view of the contradictory findings regarding the type of the phosphodiester bond synthesized by RNase T_1 (Hayashi & Egami, 1963; Podder & Tinoco, 1969; Podder, 1970; Rowe & Smith, 1971), the nature of this bond in enzymically synthesized dinucleosides was investigated by several methods.

The identity of both xanthosine phosphate isomers was established by enzymic hydrolysis of xanthosine cyclic phosphate. A solution of X>p (60 mM) in 0.2 M-citrate buffer, pH 5.4, was incubated with RNase T_1 (350 u./ml) at 37°C, and samples were withdrawn after 1, 2 and 4 days of incubation. Virtually only one xanthosine phosphate isomer was found to occur, and we assumed that it was the 3′-isomer specifically split by RNase T_1. Traces of the other isomer appeared, presumably, as a result of spontaneous hydrolysis of xanthosine cyclic phosphate (Fig. 1A). After 5 days' incubation under identical conditions, but without RNase T_1, roughly 14% of xanthosine cyclic phosphate was decomposed to a mixture of 2′ and 3′ isomers.

Xanthosine 3′-phosphate was identified as the only nucleotide after periodate oxidation of XU and XC performed according to Neu & Heppel (1964). Likewise,

only the xanthosine 3′-phosphate isomer was found after periodate oxidation irrespective whether the XU synthesis was carried out at 0° or 37°C under standard conditions. Both 2′ and 3′ isomers were differentiated by chromatography on Whatman 1 paper or/and TLC with solvent *B*. In some instances the periodate oxidation products (Fig. 1B) were first deposited on a small (11×0.7 cm) DEAE-bicarbonate column and washed with water. Xanthosine phosphate was recovered from the column with 0.12 M-triethyl-amine bicarbonate and identified by chromatography.

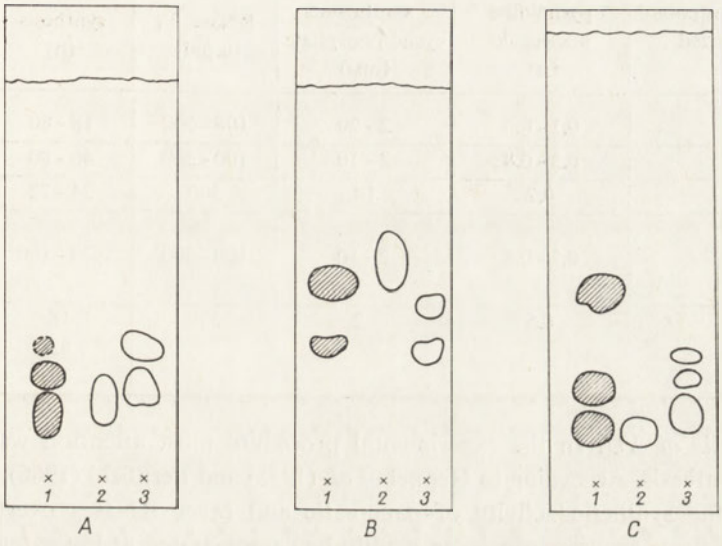

Fig. 1. TLC identification of the type of phosphodiester bond synthesized with RNase T_1. *A*, Hydrolysate of xanthosine cyclic phosphate (*1*) after 4 days of incubation with RNase T_1 under conditions given in the text; xanthosine cyclic phosphate, control (*2*); xanthosine 2′(3′)phosphate, control (*3*). *B*, XU periodate oxidation products (*1*); uracil, control (*2*); xanthosine 2′(3′)phosphate, control (*3*). *C*, Hydrolysate of XU (*1*) after 3 days of incubation with RNase T_1, under conditions given in the text. XU, control (*2*); xanthosine 2′(3′)phosphate plus xanthosine cyclic phosphate, control (*3*).

XU was also hydrolysed by RNase T_1. Two samples of a 1mM solution of XU were incubated with RNase T_1 (350 u./ml) at 37°C in (a) 0.2 M-citrate buffer, pH 5.4, and (b) 0.02 M-phosphate buffer, pH 7.0. After 2 days, in a sample of mixture (a) 75% of the dinucleoside was hydrolysed to xanthosine 3′-phosphate and uridine (Fig. 1C), whereas for mixture (b) only 25% of XU underwent hydrolysis. After alkaline hydrolysis of XU, a mixture of both xanthosine 2′ and 3′-phosphates and uridine was found.

The overall results indicate that, under conditions specified above, RNase T_1 synthesizes the 3′-5′ phosphodiester bond. Chromatographic methods showed that no xanthosine 2′-phosphate was produced after specific enzymic or chemical phosphodiester bond cleavage.

Synthesis of dinucleosides containing xanthosine and a pyrimidine component

General conditions under which dinucleoside monophosphates were synthesized using RNase T_1 are specified in Table 2.

Table 2

Enzymic syntheses of dinucleosides in 0.2 M-*citrate buffer*, pH 5.4, *at* 37°C

Type of compound synthesized	Concentration of			Duration of synthesis (h)	Yield (%)
	pyrimidine nucleoside (M)	xanthosine cyclic phosphate (mM)	RNase T_1 (u./ml)		
XU	0.1 - 0.6	2 - 20	100 - 300	18 - 80	30 - 40
XC	0.3 - 0.8	2 - 10	100 - 300	40 - 80	30 - 45
Xac	0.7	14	300	24 - 72	30
XCm $Xm^3'C$ $Xm_2^3'Cm$	0.3 - 0.6	2 - 10	100 - 300	20 - 100	30
Xm^6C	0.5	2	350	18 42 72	19 30 46

Synthesis of XU. In the experimental procedure most attention was given to the XU synthesis. According to Heppel *et al.* (1955) and Bernfield (1966), predomination of the synthetic activity of pancreatic and other RNases over hydrolytic activity of these enzymes can more readily be demonstrated at lower temperatures. The same appears true with regard to the synthesis of guanylyl(3′-5′)cytidine dinucleoside phosphate with the use of RNase T_1 as demonstrated by Rowe & Smith (1970). In the case of XU synthesis the present results clearly showed that there was no such difference; viz. for an incubation mixture of uridine (0.4 M), xanthosine cyclic phosphate (11 mM) and RNase T_1 (300 u./ml), the dinucleoside phosphate yield was 26% and 41%, respectively, after 24 and 48 h of incubation at 0°C, whereas it was 36% and 35% for both incubation periods at 37°C. The UV spectra of XU are recorded in Fig. 2. The resulting dinucleoside was quite stable at both temperatures, and only the rate of synthesis was slower at the lower temperature. The reaction products at both temperatures contained the 3′-5′ bond. All syntheses in further experiments were run at 37°C, unless otherwise indicated.

As shown in Fig. 3, the yield of XU depended on the pH, and this dependence was correlated with the degree of ionization of the xanthosine residue. The pH range investigated was 5.0-7.6, however, most syntheses were run at pH 5.4 (0.2 M-citrate buffer) because of the known acid-lability of xanthosine cyclic phosphate (Irie *et al.*, 1970).

If RNase T_1 was added to xanthosine cyclic phosphate, at the concentration of the latter exceeding 50 mM, then homodinucleotide (XXp or rather XX>p) was formed. Thus in the course of heterodinucleoside (i.e. XU) synthesis the concentra-

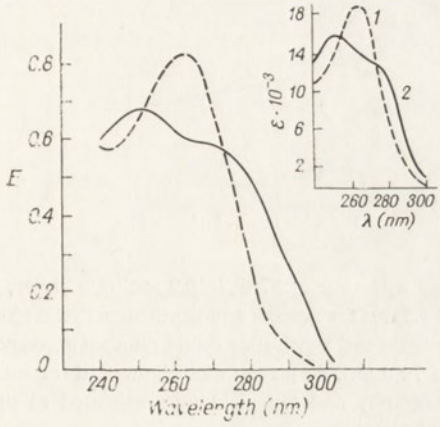

Fig. 2

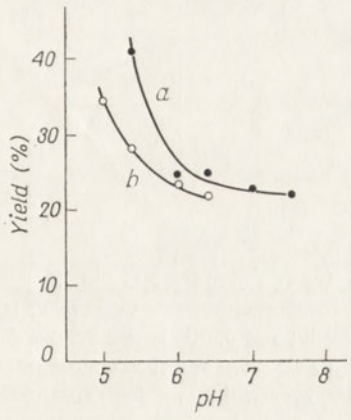

Fig. 3

Fig. 2. UV spectra of XU synthesized by RNase T_1 from xanthosine cyclic phosphate and uridine. The calculated spectra of an equimolar mixture of uridine and xanthosine are shown in the insert. Measurements were made at pH 2.0 (*1*) and pH 12.0 (*2*).

Fig. 3. Effect of pH on yield of dinucleoside monophosphate. Syntheses were run at 37°C with RNase T_1 (90 u./ml), and in *a* with uridine (0.3 M) and xanthosine cyclic phosphate (3.7 mM) in 0.2 M-cacodylic buffer for 48 h; in *b* with cytidine (0.1 M) and xanthosine cyclic phosphate (0.8 mM) in 0.2 M-citrate buffer for 44 h. The yield was estimated by chromatography as described in the text.

tion of xanthosine cyclic phosphate was kept between 5 to 20 mM, since within this concentration range it exerted no effect on the final yield, other conditions remaining constant. Neither was there any substantial change in the yield of XU when the concentration of uridine varied from 0.1 to 0.6 M. The yield depended also on the RNase T_1 concentration; it was optimal in the range 100 - 300 u./ml. Higher enzyme concentrations enhanced the hydrolysis of both the dinucleoside product and the starting (xanthosine cyclic phosphate) substrate.

With pH, temperature and concentrations constant, maximum yield of XU was attained after 40 - 50 h and persisted at this level during the next 20 - 30 h. Subsequently the yield of dinucleoside showed a slow decrease, presumably due to hydrolysis of the product and of xanthosine cyclic phosphate (Fig. 4).

Synthesis of XC. The synthesis of XC was run under standard conditions, as established for XU (Table 2 and Fig. 4). The UV spectra of chromatographically isolated XC are shown in Fig. 5. In the case of XC synthesis, as for XU synthesis, ionization of the xanthosine residue affected the yield (Fig. 3), which was the highest for non-ionized xanthosine cyclic phosphate.

Synthesis of XaC. After 72 h incubation under standard conditions no xanthosine phosphate could be demonstrated by column chromatography when arabinofuranosylcytosine was incubated with xanthosine cyclic phosphate. The UV-spectrum of XaC closely resembled those of other dinucleosides, viz. XC and its analogues (Figs. 5 and 6).

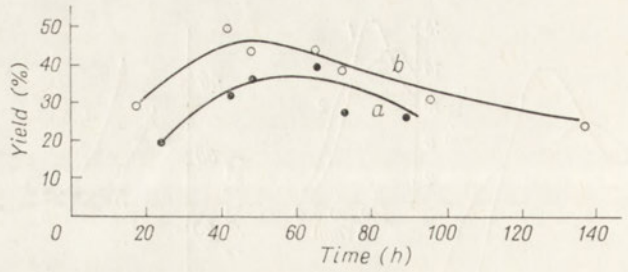

Fig. 4. The course of the XU and XC syntheses with time at 37°C, in 0.2 M-citrate buffer, pH 5.4. The starting concentrations were: *a*, 0.5 M and 4.5 mM for uridine and xanthosine cyclic phosphate, respectively; and *b*, 0.37 M and 2.3 mM for cytidine and xanthosine cyclic phosphate, respectively. RNase T_1 was used at a final concentration of 70 u./ml. At given times aliquots of the incubation mixtures were withdrawn for paper chromatography and the yield was estimated as described in the text.

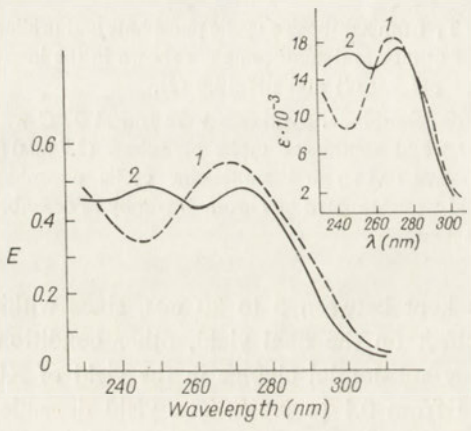

Fig. 5

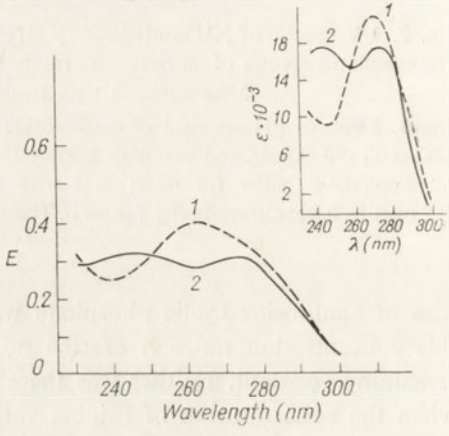

Fig. 6

Fig. 5. UV spectra of XC synthesized by RNase T_1 from xanthosine cyclic phosphate and cytidine. The calculated spectra of an equimolar mixture of cytidine and xanthosine are shown in the insert. Measurements were made at pH 2.0 (*1*) and pH 12.0 (*2*).

Fig. 6. UV spectra of Xm^6C synthesized by RNase T_1 from xanthosine cyclic phosphate and 6--methylcytidine. The calculated spectra of an equimolar mixture of 6-methylcytidine and xanthosine are shown in the insert. Measurements were made at pH 2.0 (*1*) and pH 12.0 (*2*).

Synthesis of dinucleoside phosphates with sugar-methylated cytidine derivatives at the 3′ end. 2′-*O*-methyl-, 3′-*O*-methyl- and 2′,3′-*O*-dimethylcytidine were used for the synthesis, as shown in Table 2. Because of the close R_F values of xanthosine cyclic phosphate and of the dinucleosides synthesized (Table 1), the separation of the products from substrates was possible only after previous transformation of xanthosine cyclic phosphate to xanthosine phosphate. It was expected that during this transformation (4 h at pH 1.0, at room temp.) some isomerization of the phosphodiester bond could take place. However, as reported by Griffin *et al.* (1968), for dinucleoside monophosphate UU, under analogous conditions only 0.5% of the 3′-5′ isomer undergoes transformation to the 2′-5′ isomer.

Synthesis of Xm⁶C. The UV spectra of chromatographically isolated dinucleoside phosphate are shown in Fig. 6. In a parallel experiment the synthesis of cytidylyl(3′-5′) 6-methylcytidine with the use of RNase A was carried out by Bernfield's procedure (Bernfield, 1966). Cytidine cyclic phosphate and 6-methylcytidine were dissolved in 0.1 M-tris-HCl buffer, pH 8.0, to final concentrations of 0.37 M and 0.1 M, respectively, and RNase A was added to final concentration of 30 μg/ml. The incubation mixture was kept at 0°C, and samples were withdrawn for descending paper chromatography in solvent *C*. No dinucleoside phosphate product was found even with extended incubation periods. In a control experiment the synthesis of CC, as described by Bernfield, was carried out affording a comparable yield.

Synthesis of dinucleotide diphosphates. The present results indicate that the component of the reaction forming the 3′ end of the product, provided it was a pyrimidine nucleoside, did not affect the yield. Therefore attempts were made to use pyrimidine 2′(3′) nucleotides [U2′(3′)p and C2′(3′)p] as substrates under standard conditions, as shown in Table 3.

Table 3

Enzymic syntheses of dinucleotide diphosphates in 0.2 M-*citrate buffer*, pH 5.4, *at* 37°C

U2′(3′)p (M)	C2′(3′)p (M)	X>p (mM)	RNase T_1 (u./ml)	XNp maximal yield (%)
0.3		20	150	30
	0.1	3	150	33

The yield of XUp estimated after 48, 72, 96 and 120 h showed no substantial differences, the yield of XCp was determined only after 72 h of incubation. Since paper chromatographic separation of the substrates and products presented some difficulties, samples withdrawn for chromatography were pre-incubated with bacterial alkaline phosphatase, and only then applied to paper and run in solvent *A*. Thus, the XNp product was quantitatively determined as its XN derivative.

Synthesis of dinucleosides containing xanthosine and a purine component

All attempts to synthesize the XN product, using a purine nucleoside (xanthosine, inosine, adenosine, guanosine or 2′-*O*-methyladenosine) as N, were unsuccessful. There was no product formation under various conditions of temperature (0° and 37°C), pH (5.4 - 7.0), concentrations of substrates and of the enzyme. Obviously the low solubility of purine nucleosides is one of the limiting factors; however, even in the case of 2′-*O*-methyladenosine, in 0.3 M solution, no synthesis could be observed.

Obviously, homodinucleoside phosphate (XX) was the most interesting product expected. Therefore, attention was given to development of conditions permitting

this synthesis. In addition to the above-mentioned modifications of the standard conditions, attempts were made to increase the solubility of xanthosine by addition of pyridine, dimethylformamide or dimethylsulphoxide to the incubation medium (Zhenodarova, 1970). Paper chromatography showed that no XX was formed up to 5 days incubation.

Xanthosine phosphate is known to be an RNase T_1 inhibitor; our failure to synthesize XXp, starting with xanthosine 2′(3′)phosphate and xanthosine cyclic phosphate as substrates, confirmed this fact (Mohr & Thach, 1969; Campbell & T'so, 1971). Therefore, in the present experiments the XX derivatives could be synthesized only with RNase T_1 acting on xanthosine cyclic phosphate itself.

The following conditions were found to be satisfactory: xanthosine cyclic phosphate (0.4 - 0.7 M solution, 20 mg for one preparative run) in 0.2 M-citrate buffer, pH 5.5, and RNase T_1 (300 u./ml) were incubated for 4 days at 37°C. After 24 h incubation, paper chromatography in solvent *A* revealed new spots. The reaction was stopped after 90 h incubation by diluting the mixture to 10 ml with water and adjusting the pH to 7.5 with concentrated ammonia. This solution was applied to a DEAE-cellulose (bicarbonate form) column (20×1.2 cm), washed with water and eluted with linear triethylamine bicarbonate (TEA·CO_2) gradient, followed by step-wise elution with 0.7 M and 1.0 M-TEA·CO_2. Fractions of 5 ml were collected at a flow rate of 2.3 ml/min.

In the parallel experiment, after 90 h incubation the reaction mixture was diluted to 1 ml and incubated at pH 1.0 for 4 h at room temperature for opening of cyclic phosphates. The subsequent steps of the procedure were analogous as above; the comparative elution profiles are shown in Fig. 7. Peaks *1* and *2* were identified by paper chromatography as xanthosine cyclic phosphate and xanthosine 2′(3′)phosphate, respectively.

Fractions corresponding to peaks *3* and *4* when subjected to alkaline hydrolysis (0.3 M-KOH, 37°C, 18 h) were converted exclusively to xanthosine 2′(3′)phosphate, as identified by paper chromatography.

Fractions corresponding to peak *4* treated with bacterial alkaline phosphatase (pH 8.8, 37°C, 24 h) and then subjected to alkaline hydrolysis in 0.3 M-KOH yielded equimolar amounts of xanthosine and xanthosine 2′(3′)phosphate which could be separated by paper chromatography and quantitatively determined after elution.

Fractions corresponding to peak *4* dephosphorylated with bacterial alkaline phosphatase and rechromatographed on a DEAE-cellulose column under the above described conditions were eluted at a TEA·CO_2 concentration of 0.16 - 0.18 M. When subjected to alkaline hydrolysis this substance was found to consist of equimolar mixture of xanthosine and xanthosine 2′(3′)phosphate. According to that result this substance was identified as dinucleoside monophosphate (XX).

The presented data allowed identification of peak *3* as dinucleoside diphosphate with cyclic 2′,3′-terminal phosphate (XX›p), and of peak *4* as dinucleoside diphosphate (XXp).

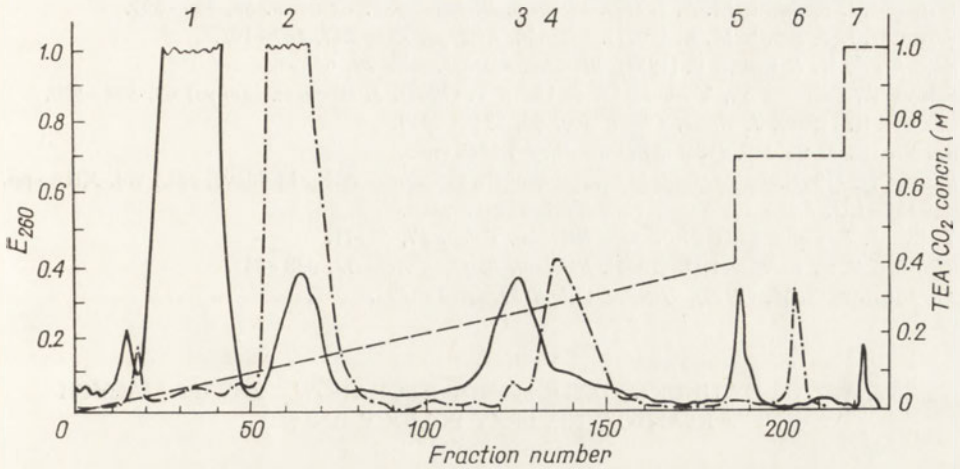

Fig. 7. DEAE-cellulose column chromatography: (——) of the products of RNase T_1 action on xanthosine cyclic phosphate; (-·-·-), of a similar incubation mixture which, after completion of the enzymic reaction, was treated with 0.1 N-HCl for opening of cyclic phosphates. Conditions of chromatography and peak identification are given in the text. Peaks *1* - *4* correspond to xanthosine cyclic phosphate, xanthosine 2′(3′)phosphate, dinucleoside diphosphate with cyclic 2′,3′-terminal phosphate (XX>p), and dinucleoside diphosphate (XXp), respectively.

Peaks eluted with 0.7 and 1.0 M-TEA · CO_2 were not identified; peaks *5* and *6* correspond presumably to trinucleoside triphosphates.

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SYNTEZA DWUNUKLEOZYDOMONOFOSFORANÓW ZAWIERAJĄCYCH KSANTOZYNĘ, PRZY POMOCY RNazy T_1

Streszczenie

1. Wykazano, że RNaza T_1 zdolna jest syntetyzować dwunukleozydomonofosforany typu XN. Substratami reakcji były cykliczny fosforan ksantozyny i niektóre pirymidynowe nukleozydy i nukleotydy. Ustalono warunki maksymalnej wydajności reakcji.

2. Produkty reakcji wyizolowano chromatograficznie i przeprowadzono ich identyfikację metodami spektrofotometrycznymi i chromatograficznymi. Udowodniono, że enzym syntetyzuje wiązanie fosfodwuestrowe typu 3′-5′ z wysoką wydajnością (do 45%).

3. Wszystkie substraty i produkty można łatwo odzyskać po reakcji i ponownie wykorzystywać.

4. Opisana metoda może znaleźć zastosowanie w preparatyce dwunukleozydomonofosforanów zawierających terminalną 5′-ksantozynę. Otrzymane związki mogą być użyte do badań konformacji oligo- i polinukleotydów.

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THE EFFECT OF SODIUM AND SOME OTHER ALKALI CATIONS ON CALCIUM TRANSPORT IN RAT DUODENUM

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1. The everted gut-sac technique was used to study the influence of sodium and some other alkali cations on the mechanism of calcium transport in rat duodenum. 2. The active expulsion of calcium from the mucosa toward the serosa was presumably dependent on the presence of sodium in the incubation medium whereas the movement of calcium across the mucosal surface did not require sodium. 3. Both the uptake and expulsion of calcium were affected by low temperature, anaerobiosis, KCN and dinitrophenol. Li^+, K^+, Rb^+, Cs^+ and NH_4^+ ions did not replace sodium and inhibited the sodium-dependent transport of calcium. All these cations affected the process of calcium uptake at 37°C but had no effect at 5°C.

The majority of available evidence supports the idea that in rat duodenum calcium moves up an electrochemical potential gradient by a specific, energy-dependent, saturable, cation-oriented process (Wasserman & Taylor, 1969). However, the detailed mechanism of this active transport still remains unclear. Studies *in vitro* using the everted gut sac technique showed that active translocation of calcium across the duodenal wall seems to involve at least two distinct steps (Schachter *et al.*, 1960, 1966; Michalska *et al.*, 1972): 1, uptake at the mucosal surface, and 2, transport to or toward the serosal surface, i.e. the egress of calcium from the mucosal cells at the basal-lateral membrane. Both steps are rate-limiting and were found to be affected by vitamin D administration (Schachter *et al.*, 1966; Hashim & Clark, 1969; Adams & Norman, 1970; Adams *et al.*, 1970). The results obtained by Schachter *et al.* (1966) indicate that the active step of calcium transport, i.e. transfer against the concentration gradient, concerns the expulsion step, whereas the uptake represents a facilitated diffusion. There is, however, some contradictory evidence suggesting that an active mechanism mediates also the uptake of calcium (Hashim & Clark, 1969).

Sodium ion, previously thought to have no influence on calcium transport (Harrison & Harrison, 1963; Wasserman & Taylor, 1963) has been recently found

to be required (Martin & DeLuca, 1969; Birge *et al.*, 1972; Wróbel *et al.*, 1973). It was also suggested that the basal-lateral membrane of the mucosal cell is most likely the site of sodium action (Martin & DeLuca, 1969; Adams & Norman, 1970). Some other alkali cations like potassium or lithium did not substitute for sodium and were found to be partially inhibitory for calcium translocation in the intestine (Schachter *et al.*, 1960; Wasserman & Taylor, 1963).

The results of the present work suggest that both the uptake and expulsion steps of calcium transport in rat duodenum are metabolically dependent processes. The expulsion step is presumably dependent on sodium whereas calcium uptake does not require sodium and is affected by some other alkali cations.

MATERIAL AND METHODS

Reagents. All the reagents were analytical grade products supplied by Biuro Obrotu Odczynnikami Chemicznymi (Gliwice, Poland). $^{45}CaCl_2$ (spec. act. 312 mCi/mmol) was from Biuro Dystrybucji Izotopów (Warszawa, Poland).

Animals. Wistar rats of both sexes 5 - 6 weeks old (body weight 70 - 80 g) were fed a standard laboratory diet. The animals were fasted for 16 h, then killed by a blow on the head followed by decapitation.

Preparation of everted duodenal sacs. Immediately after decapitation of the animal, 5 cm of the intestine proximal to the pyloric valve was excised, rinsed with an ice-cold isotonic solution of glucose and everted using a plastic rod. One end of the segment was tied with a ligature and the serosal compartment was filled with 0.5 ml of incubation medium using a syringe fitted with a blunt needle.

Incubation. The basal incubation medium ("sodium free") consisted of 50 mM-imidazole-HCl buffer, pH 7.4, 20 mM-glucose, 0.8 mM-Na_2HPO_4, 0.4 mM-$CaCl_2$ with the addition of ^{45}Ca (20 μCi/100 ml) and 240 mM-mannitol. Depending on the experiment, instead of mannitol NaCl, KCl, LiCl, RbCl, CsCl or NH_4Cl was added in an amount that maintained the osmotic pressure of the incubation medium. The sac was placed in 25 ml Erlenmeyer flask containing 5 ml of incubation medium and incubated with shaking for 90 min at 37°C under a continuous flow of oxygen. Following incubation the internal serosal fluid was collected quantitatively and the radioactivity was determined both in the internal and external medium.

Measurement of ^{45}Ca content in the mucosa. After removal of the internal fluid the sac was rinsed with 0.9% NaCl solution and blotted. Mucosal tissue was gently scraped off, then dried for 24 h at 110°C, weighed, ashed in a muffle furnace, the residue dissolved in concentrated hydrochloric acid, and the radioactivity determined.

Analytical procedures. Sodium was estimated by flame photometry. ^{45}Ca radioactivity was determined in a liquid scintillation counter (Nuclear Chicago Mark I) using a scintillation fluid containing 4.0 g of PPO and 100 mg of POPOP per 1 litre of toluene - methanol (3 : 7, v/v).

Presentation of results. Active transport of calcium was calculated from the difference between the content of ^{45}Ca in the serosal fluid after incubation, and

its initial content; it was expressed as net accumulation of calcium (nmol/sac/90 min).

Uptake of ^{45}Ca from the mucosal fluid was expressed as percentage of the initial amount of ^{45}Ca in this fluid.

RESULTS

Calcium accumulation against the concentration gradient in the serosal compartment of everted duodenal sac was dependent on the presence of sodium ion in the incubation medium (Table 1). The lowest concentration of Na^+ required for a fully efficient calcium transport was 40 mM when sodium chloride was placed both sides of the intestinal wall. It is of interest that the same values of active accumulation of calcium were observed when 80 or 120 mM-sodium chloride was present initially in the serosal medium only.

Table 1

Effect of sodium chloride on calcium transport by everted duodenal sac

Incubation conditions as described in Materials and Methods, except that NaCl was added either both sides of the duodenal wall or on the serosal side only. The values are means of 4 - 6 experiments ±S.D.

NaCl concentration (mM)		Net accumulation of ^{45}Ca in serosal fluid (nmol/sac/90 min)
mucosal fluid	serosal fluid	
0	0	0
20	20	5± 0.4
0	20	0
40	40	230± 8.5
0	40	113± 5.3
60	60	231±37
0	60	130±13
80	80	227±15.2
0	80	224±19.7
120	120	232±20.3
0	120	228±22.6

Sodium ion placed in the serosal compartment moved freely through the duodenal wall and after 90 min equilibration took place (Table 2). At 120 mM initial NaCl concentration in the serosal fluid, the concentration of Na^+ in the mucosal fluid after equilibration did not reach 20 mEq/l, a concentration which, when present on both sides of the everted sac, was not sufficient to promote active transport of calcium (Table 1). Thus, sodium at higher concentration seems to be an important factor for the serosal part of calcium transport. On the assumption that the transport mechanism which mediates the active expulsion of calcium from the mucosal cells is dependent on the presence of sodium, whereas the mechanism mediating the uptake of calcium at the brush border side of the cells does not require sodium,

a higher level of ^{45}Ca might be expected to be present in the mucosa of the sac incubated in the absence of sodium as compared with the sac incubated in its presence. This supposition was confirmed by the results of experiments shown in Table 3. The content of ^{45}Ca in the mucosa was by 40% lower in the presence of sodium than in its absence, whereas the uptake from the mucosal fluid was the same.

Table 2

Equilibration of Na^+ *concentration following incubation of everted sacs with* NaCl *added to the serosal fluid*

Incubation conditions as described in Materials and Methods. Values are the means of a typical experiment run in duplicate.

Na^+ concentration (mEq/l)				
before incubation		after incubation		Theoretical calculation of equilibration
mucosal fluid	serosal fluid	mucosal fluid	serosal fluid	
1.6	1.6	2.8	4.2	1.6
1.6	121.6	13.2	20.0	12.6
1.6	61.6	7.8	13.8	7.1

Table 3

Effect of alkali and NH_4^+ *cations on* ^{45}Ca *uptake and expulsion by the everted duodenal sac*

Incubation conditions as described in Materials and Methods. Initial content of ^{45}Ca in mucosal fluid was 7090 c.p.m./0.1 ml. Values are the means of a typical experiment run in duplicate.

Addition to the basal medium	Uptake of ^{45}Ca (% of initial amount)	Content of ^{45}Ca in mucosa		Net accumulation of ^{45}Ca in serosal fluid (nmol/sac/90 min)
		c.p.m./10 mg dry tissue	% of control	
None (control)	40	19 700	(100)	0
NaCl 70 mM	41	11 700	59	210
KCl 70 mM	25	11 100	56	0
LiCl 70 mM	22	10 400	53	0
CsCl 70 mM	13	8 800	44	0
NH_4Cl 70 mM	15	10 200	51	0

No calcium accumulation in the serosal fluid was observed when 70 mM potassium, lithium, ceasium or ammonium ions replaced sodium (Table 3), and in the presence of sodium they inhibited the expulsion of calcium (Table 4). Potassium at lower concentration was less effective than other ions (Fig. 1). Simultaneously these cations inhibited the uptake of ^{45}Ca from the mucosal fluid both in the presence and absence of sodium (Table 4).

All these data support the participation of sodium in the expulsion of calcium from the mucosal cells at the basal membranes and indicate that sodium is not

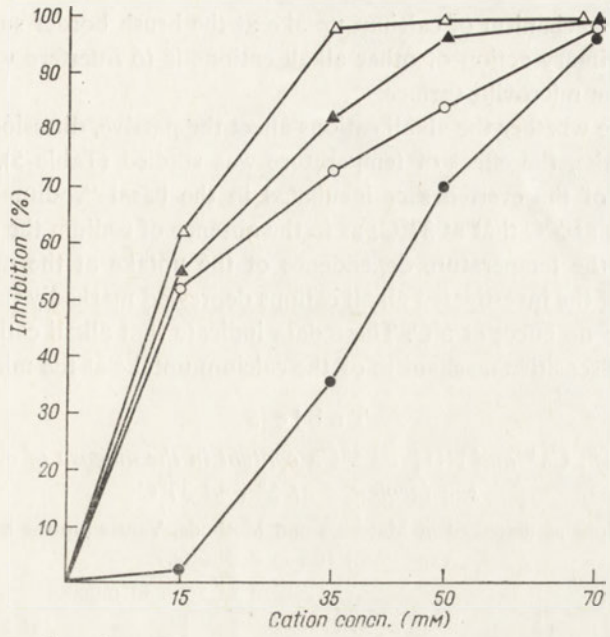

Fig. 1. Inhibitory effect of ●, KCl; ▲, LiCl; ○, CsCl; and △, NH_4Cl on calcium transport by everted sacs incubated in the presence of sodium. Incubation conditions as described in Materials and Methods except that the incubation medium contained 50 mM-NaCl.

Table 4

Effect of K^+, Li^+, Rb^+, Cs^+ and NH_4^+ *on the* ^{45}Ca *uptake and expulsion by everted duodenal sac incubated in the presence of sodium*

Experimental conditions as described in Materials and Methods. Initial content of ^{45}Ca in mucosal fluid was 7800 c.p.m./0.1 ml. Values are the means of a typical experiment run in duplicate.

Additions to the basal medium	Uptake of ^{45}Ca from mucosal fluid (% of initial amount)	Content of ^{45}Ca in mucosa		Net accumulation of ^{45}Ca in serosal fluid (nmol/sac/90 min)
		c.p.m./10 mg dry tissue	% of control	
None	56	27 803	196	0
NaCl 50 mM (control)	52	41 188	(100)	284
NaCl 50 mM KCl 70 mM	24	10 136	71	0
NaCl 50 mM LiCl 70 mM	26	11 280	78	0
NaCl 50 mM RbCl 70 mM	20	8 990	63	0
NaCl 50 mM CsCl 70 mM	23	8 250	58	0
NaCl 50 mM NH_4Cl 70 mM	16	10 700	75	0

required in the mechanism of calcium uptake at the brush border surface. It seems also that the primary action of other alkali cations is to interfere with the uptake of calcium at the microvilli surface.

To determine whether the alkali cations affect the passive, diffusional mechanism of calcium uptake, the effect of temperature was studied (Table 5). ^{45}Ca content of the mucosa of the everted sacs incubated in the basal "sodium-free" medium was much lower at 5°C that at 37°C; as in the absence of sodium the exit of calcium did not occur, the temperature dependence of the uptake at the mucosal surface is evident. All of the investigated alkali cations depressed markedly the ^{45}Ca content at 37°C but had no effect at 5°C. These data indicate that alkali cations affect only the temperature-sensitive mechanism of the calcium uptake at the microvilli surface.

Table 5

Effect of K^+, Li^+, Cs^+ *and* NH_4^+ *on* ^{45}Ca *content in the mucosa of everted duodenal sac incubated at* 5° *and* 37°C

Incubation conditions as described in Materials and Methods. Values are the means of a typical experiment run in duplicate.

Additions to the basal medium	Content of ^{45}Ca in mucosa			
	at 37°C		at 5°C	
	c.p.m./10 mg dry tissue	% of control	c.p.m./10 mg dry tissue	% of control
None (control)	12 980	(100)	6 750	(100)
KCl 70 mM	7 528	58	7 020	104
CsCl 70 mM	6 749	52	6 277	93
LiCl 70 mM	7 529	58	7 425	110
NH_4Cl 70 mM	6 100	47	5 737	85

Schachter *et al.* (1966) suggested that in duodenum the calcium uptake step is not energy dependent. In connection with this suggestion in our experiments we have studied the effects of cold, anoxia and some respiratory poisons on calcium uptake. In the presence of sodium (Table 6) low temperature, 2,4-dinitrophenol,

Table 6

Effect of temperature, anoxia and some metabolic poisons on ^{45}Ca *content in the mucosa and on calcium transport by everted sac incubated in sodium-containing medium*

Incubation medium contained 50 mM-NaCl. Other conditions as described in Materials and Methods. Values are the means of a typical experiment run in duplicate.

Incubation conditions	Content of ^{45}Ca		Net accumulation of ^{45}Ca in serosal fluid (nmol/sac/90 min)
	c.p.m./10 mg dry tissue	% of control	
Control	5 410	(100)	230
at 5°C	4 950	91	0
N_2 atmosphere	4 909	90	0
KCN 5 mM	4 820	89	0
DNP 1 mM	6 040	112	0

Table 7

Effect of temperature, anoxia and some metabolic poisons on ^{45}Ca content in the mucosa of everted sac incubated in "sodium-free" medium

The experiments were performed using basal "sodium-free" medium, other conditions as described in Materials and Methods. Values are the means of a typical experiment run in duplicate.

Incubation conditions	Content of ^{45}Ca in mucosa	
	c.p.m./10 mg dry tissue	% of control
Control	11 260	(100)
at 5°C	5 635	50
N_2 atmosphere	4 670	40
KCN 5 mM	5 145	45
DNP 1 mM	4 715	42

potassium cyanide and a nitrogen atmosphere inhibited completely the active transport of calcium. In the absence of sodium (Table 7), when no expulsion of calcium occurred and the absorbed ^{45}Ca accumulated in the mucosa, these agents depressed the content of calcium in the mucosa. The above results point to energy dependence of calcium uptake at the mucosal surface of the duodenum.

DISCUSSION

The presented data confirm the earlier results of Martin & DeLuca (1969) and Birge *et al.* (1972) that the net movement of calcium across the everted duodenal sac is dependent on the presence of sodium in the incubation medium, and provide evidence for the view expressed by those authors that sodium is required not for the calcium uptake step but for its expulsion from the mucosal cells. Accumulation of calcium in the serosal medium occurred only in the presence of sodium whereas ^{45}Ca uptake from the mucosal fluid was essentially the same in the presence or absence of sodium.

Our experiments show that K^+, Li^+, Rb^+, Cs^+ and NH_4^+ are effective inhibitors of the active transport of calcium, probably acting primarily on the uptake of calcium by the mucosa. As the investigated alkali cations did not affect calcium uptake by the mucosa at 5°C, it is evident that they affect the temperature-dependent mechanism of calcium uptake. It should be noted that in experiments with artificial membranes Massey & McColloch (1971) demonstrated a competition between alkali cations and calcium for binding to membranes. Also, allosteric inhibition of calcium uptake and adenosine triphosphatase activity of skeletal muscle microsomes by alkali ions was found to occur (de Meis, 1971). Taking into account the suggestions of Martin *et al.* (1969), Norman *et al.* (1970) and Krawitt (1972) that Ca^{2+}-dependent

ATPase and alkaline phosphatase may play a role in the uptake of calcium at the brush border surface of the intestinal cells, it seems possible that alkali cations might affect some enzymic processes. It should be emphasized that the presented evidence does not exclude the possibility that alkali cations also affect the mechanism of calcium egress.

Since the mechanism which mediates the egress of calcium at the serosal side of mucosal cells of the duodenum is not operative in the absence of sodium, the content of ^{45}Ca in the mucosa under these conditions reflects the process of calcium uptake. Thus the observations which show that low temperature, anaerobiosis, KCN and 2,4-dinitrophenol markedly depressed the content of ^{45}Ca in the mucosa of the everted sacs incubated in the absence of sodium, indicate that calcium uptake is metabolically and energy dependent. This conclusion is in accordance with the studies of Hashim & Clark (1969) who suggested an active process of calcium uptake by isolated mucosal cells. In agreement with Schachter *et al.* (1966) we found that, when sodium was present, anaerobiosis and respiratory poisons had no effect on the ^{45}Ca content in the mucosa. However, these results could be interpreted as being due to inhibition of both the uptake and expulsion steps of the mechanism mediating the active transport of calcium.

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WPŁYW SODU I NIEKTÓRYCH INNYCH ALKALICZNYCH KATIONÓW NA TRANSPORT WAPNIA W DWUNASTNICY SZCZURA

Streszczenie

1. Metodą odwróconych woreczków jelitowych badano wpływ sodu i niektórych innych alkalicznych kationów na mechanizm transportu wapnia w dwunastnicy szczura.

2. Aktywne wyrzucanie wapnia ze śluzówki w kierunku surowicówki zależało od obecności sodu w środowisku inkubacyjnym, podczas gdy przechodzenie wapnia przez powierzchnię śluzówkową sodu nie wymagało.

3. Zarówno pobieranie jak i wyrzucanie wapnia było hamowane przez niską temperaturę, warunki beztlenowe, KCN oraz dwunitrofenol.

4. Jony K^+, Li^+, Rb^+, Cs^+ i NH_4^+ nie zastępowały sodu oraz hamowały transport wapnia zależny od sodu. Wszystkie te kationy wpływały na proces pobierania wapnia w 37°C nie mając wpływu w 5°C.

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THE SUBUNIT STRUCTURE OF RABBIT LIVER *p*-HYDROXYPHENYLPYRUVATE HYDROXYLASE

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Evidence is presented that pHPP hydroxylase from rabbit liver is constituted of subunits. The enzyme of 150 000 mol. wt. was disaggregated to 70 000 subunits, then to inactive 40 000 subunits. The activity of 70 000 subunit was half that of the native enzyme. By polyacrylamide gel electrophoresis it was shown that 70 000 subunits are charge isomers and 40 000 subunits charge and size isomers. The results of experiments with ferrous ion and SH-containing agents suggested that Fe^{2+} is necessary for maintenance of the tetrameric form of the enzyme and its full activity. Free SH groups prevented the oxidation of ferrous ion.

Goswami (1964) demonstrated reversible inhibition of *p*-hydroxyphenylpyruvate hydroxylase[1] (EC 1.14.2.2) by *o*-phenanthroline and suggested the requirement of a reduced metal ion, probably iron, for maintaining the enzyme activity.

The presented results prove that the effect of iron on pHPP hydroxylase is due to its involvement in subunit structure of this enzyme. Removal of iron leads to reversible disaggregation of the enzyme and concomitant loss of the activity.

MATERIALS AND METHODS

Special reagents. 2,6-Dichlorophenolindophenol (Biuro Obrotu Odczynnikami Chemicznymi, Gliwice, Poland), *p*-hydroxyphenylpyruvic acid, *N,N'*-methylenebisacrylamide, sodium dodecyl sulphate, cytochrome *c* from horse heart, rabbit γ-globulin fraction II, fumarase from pig heart (Koch-Light, Colnbrook, Bucks., England), acrylamide, EDTA, *o*-phenanthroline (B.D.H., Poole, Dorset, England), riboflavin, 5,5'-dithiobis-(2-nitro)benzoic acid (Sigma, St. Louis, Mo., U.S.A.), reduced glutathione (Nutr. Biochem. Corp., Cleveland, Ohio, U.S.A.), dithiothreitol (Calbiochem, Los Angeles, Calif., U.S.A.), Sephadex G-25, G-50, G-150, G-200,

[1] Abbreviations used: pHPP, *p*-hydroxyphenylpyruvate; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitro)benzoic acid; SDS, sodium dodecyl sulphate; GSH, reduced glutathione; DCPP, 2,6-dichlorophenolindophenol.

DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden), Whatman DEAE-cellulose (Balston, Maidston, Kent, England), *N,N,N',N'*-tetramethylethylenediamine and tris (Fluka AG, Buchs, Switzerland), $Fe(NH_4)_2(SO_4)_2 \cdot 7H_2O$ (Merck, Darmstadt, G.F.R.), ribonuclease A from bovine pancreas, bovine serum albumin fraction V (Pentex, Kankakee, Ill., U.S.A.).

Crude enzyme preparation. Rabbit liver was homogenized with 3 vol. of 0.15 M-KCl in 0.002 M-NaOH in a Waring blendor for 2 min (Goswami, 1964). The homogenate was centrifuged for 20 min at 20 000 *g*, the sediment was discarded and the supernatant centrifuged again at 105 000 *g* for 2 h at 0°C. The protein of the 105 000 *g* supernatant was fractionated with ammonium sulphate at pH 7.3. The precipitate at 0.4 - 0.7 saturation containing all pHPP hydroxylase activity was dissolved in 0.01 M-Na,K-phosphate buffer, pH 7.3, and desalted on Sephadex G-50.

Enzyme activity was determined according to Zannoni & La Du (1959). The standard incubation mixture contained in a final volume of 2.6 ml: 20 μmol of GSH, 25 μg of DCPP, enzyme preparation, 0.1 M-Na,K-phosphate buffer, pH 6.5, and 1.6 μmol of pHPP. After 20 min incubation at 37°C the activity was assayed by measuring the decrease of substrate by the enol (aromatic) borate tautomerase method according to Lin *et al.* (1958). The activity was expressed as μmol of substrate decomposed/mg of protein/20 min. When the effect of ferrous ion was tested, $Fe(NH_4)_2(SO_4)_2$ was used.

Disc electrophoresis. Electrophoresis was performed on 7.5% polyacrylamide gel (0.5×6 cm) at pH 9.5, according to Davis (1964). About 15 μg of the protein in 20% sucrose was layered directly on the top of the spacer gel and the electrophoresis was carried out at 3 mA per tube for 60 min. Gels were stained with 0.5% Amido Black in 7% acetic acid, and destained by washing with 7% acetic acid. For measurement of the enzyme activity the gel after electrophoresis was sliced into 2 mm discs and eluted with 0.3 ml of 0.01 M-Na,K-phosphate buffer, pH 7.3, for 1 h at 4°C.

Molecular size determination was made on polyacrylamide gel according to Hedrick & Smith (1968).

Approximate molecular weight was estimated by Sephadex G-200 and G-150 gel filtration according to Andrews (1964). The column (1.5×40 cm) was equilibrated with 0.01 M-Na,K-phosphate buffer, pH 7.3. The elution volume of proteins was assayed at 280 nm and of the enzyme by the activity measurements.

Analytical centrifugation. Sedimentation coefficients were determined at 20°C with a Beckman Spinco Model E ultracentrifuge equipped with a phase plate schlieren diaphragm.

Column chromatography. Columns of DEAE-Sephadex A-50 and of DEAE-cellulose (2.5×30 cm) were equilibrated with 0.05 M-NaCl in 0.01 M-Na,K-phosphate buffer, pH 7.3, and proteins were eluted with a continuous gradient of 0.1 - 0.3 M-NaCl in 0.01 M-Na,K-phosphate buffer, pH 7.3. Fractions of 3 ml were collected.

Protein was determined according to Lowry *et al.* (1951) with bovine serum albumin as a standard.

Free thiol groups were determined by the method of Ellman (1959) in the modification of Butterworth *et al.* (1967). Protein, 0.25 mg in 1 ml of 0.1 M-tris buffer, pH 7.5, was allowed to react with 0.1 ml of 0.01 M-DTNB. After 30 min at room temperature the sample was applied to Sephadex G-25 column (1×20 cm) and the thionitrophenylated protein eluted with 0.1 M-tris buffer, pH 7.5. The eluates were pooled and an excess of reducing agent (DTT) was added to cleave the mixed disulphide bonds, and after 30 min the liberated thionitrophenylate anion was measured spectrophotometrically at 412 nm. For calculation the molecular extinction coefficient of 13 600 was used. To the parallel sample SDS was added to a final concentration of 0.5% and after 30 min at room temperature an excess of DTT was added. Thereafter the SH groups were determined as described above.

Ferrous ion was estimated with *o*-phenanthroline as described by Massey (1960).

RESULTS

Disaggregation of native pHPP hydroxylase

Activity of the hydroxylase in crude homogenate, 105 000 *g* supernatant and after ammonium sulphate fractionation was not affected by Fe^{2+} (Table 1).

On Sephadex G-150 gel filtration three protein peaks were obtained (Fig. 1) two of which showed hydroxylase activity on direct measurement, while proteins of the third peak were active only after addition of Fe^{2+}. The enzyme protein of

Table 1

Purification of pHPP hydroxylase

Specific activity was expressed as μmol of substrate/mg of protein /20 min. Approximate molecular weight was determined by gel filtration method (Fig. 2).

	Stage of preparation	Specific activity		Molecular weight	Purification factor
		without Fe^{2+}	with 1 mM-Fe^{2+}		
1	Homogenate	0.006	0.006		—
2	105 000 *g* supernatant	0.050	0.050	150 000	8
3	Desalted ppt. at 0.4 - 0.7 $(NH_4)_2SO_4$ sat.	0.095	0.095	150 000	16
4	Sephadex G-150				
	peak *I*	0.42	0.42	150 000	70
	peak *II*	0.18	0.32	70 000	53
	peak *III*	0.00	0.18	40 000	30
	Peak *I*				
5	DEAE-Sephadex A-50	0.98	1.00	150 000	170
6	DEAE-cellulose	1.03	1.80	40 000	300
	Peak *II*				
5	DEAE-Sephadex A-50	0.48	0.90	70 000	150
6	DEAE-cellulose	1.07	1.60	40 000	270

peak *I* on Sephadex G-200 showed a molecular weight of approximately 150 000 (Fig. 2). As the enzyme activity present in the 105 000 *g* supernatant also emerged in the volume corresponding to 150 000 mol. wt., it was assumed that peak *I* contains the native enzyme.

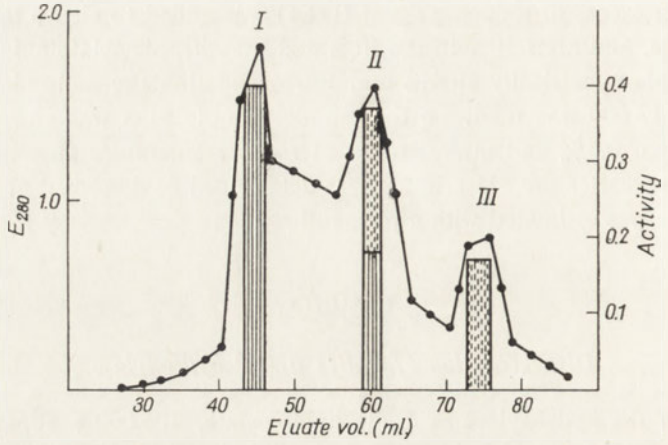

Fig. 1. Sephadex G-150 gelfiltration of pHPP hydroxylase. Desalted preparation (200 mg of protein) was applied to the Sephadex G-150 column equilibrated with 0.01 M-Na,K-phosphate buffer, pH 7.3. The same buffer was used for elution and 3 ml fractions were collected. Enzymic activity was determined without (shaded area), and with the addition of 1 mM-Fe^{2+} (dotted area) and expressed as the decrease of substrate in μmol/mg of protein; ●, protein.

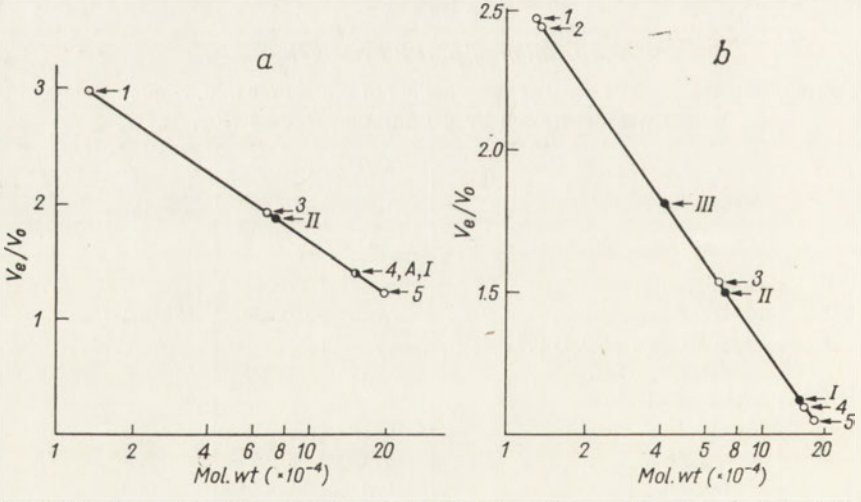

Fig. 2. Molecular weight determination of pHPP hydroxylase and its subunits by gel filtration on *a*, Sephadex G-200, and *b*, Sephadex G-150. The Sephadex columns (1.5×40 cm) were equilibrated with 0.01 M-Na,K-phosphate buffer, pH 7.3. The following standard proteins (3 mg each) dissolved in the same buffer were used: *1*, cytochrome *c*, mol. wt. 13 000; *2*, ribonuclease A, mol. wt. 13 700; *3*, bovine serum albumin, mol. wt. 67 000; *4*, rabbit γ-globulin, mol. wt. 150 000; *5*, fumarase, mol. wt. 190 000. *A*, crude homogenate; *I*, *II*, *III*, peaks after Sephadex G-150 (Fig. 1) gel filtration.

The approximate molecular weight of the enzyme in peak *II* was 70 000, and its specific activity was half that of the native enzyme (Table 1). The enzyme was activated by iron (Fig. 3) and in the presence of 1 mM-Fe^{2+} the activity almost reached that of the 150 000 native enzyme.

The enzyme of peak *III* had the molecular weight of 40 000 and was active only in the presence of 1 mM-Fe^{2+}; its specific activity was then the same as that of the 70 000 subunit without the addition of ferrous ion (Table 1). The 40 000 enzyme fraction was unstable and after a few hours at 4°C was irreversibly precipitated.

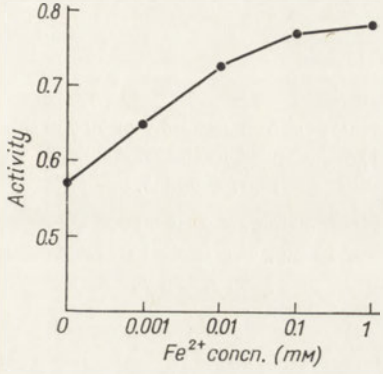

Fig. 3. Effect of Fe^{2+} addition on the enzymic activity of 70 000 subunit. The standard incubation mixture contained 1.6 mg of protein of peak *II* after Sephadex G-150 gel filtration and varying concentration of Fe^{2+}. The activity is expressed as decrease of substrate in μmol/sample.

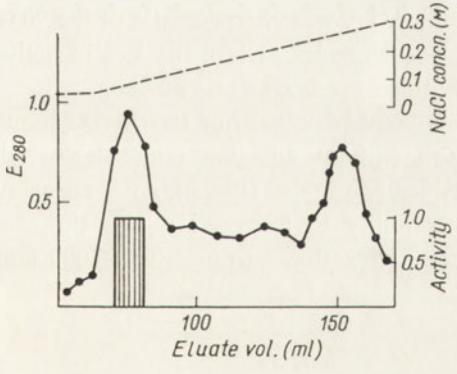

Fig. 4

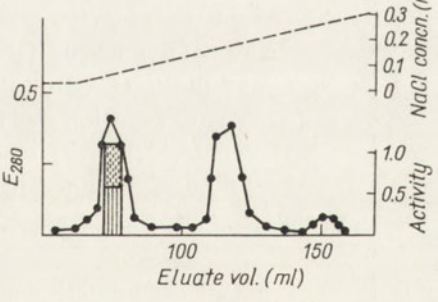

Fig. 5

Fig. 4. DEAE-Sephadex A-50 column chromatography of pHPP hydroxylase. Protein, 80 mg of peak *I* from Sephadex G-150 (Fig. 1), was applied to the DEAE-Sephadex A-50 column equilibrated with 0.05 M-NaCl. For elution of protein, a continuous gradient of 0.1 - 0.3 M-NaCl in 0.01 M-Na, K-phosphate buffer, pH 7.3, was used. Fractions of 3 ml were collected. The activity was measured without the addition of Fe^{2+}. ●, Protein; shaded area, enzymic activity (μmol of substrate decomposed/mg of protein); — — —, NaCl concentration gradient.

Fig. 5. DEAE-cellulose chromatography of pHPP hydroxylase. Protein, 40 mg, obtained after DEAE-Sephadex A-50 chromatography (Fig. 4) was applied to DEAE-cellulose column. Dotted area, the increase of enzymic activity after addition of 1 mM-Fe^{2+}.

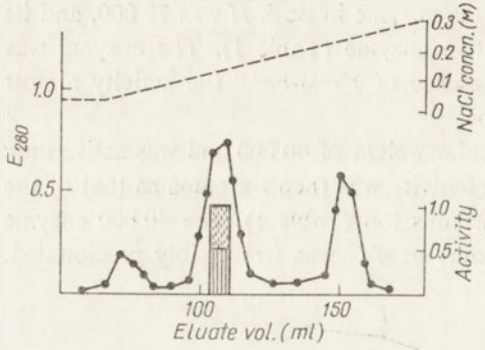

Fig. 6

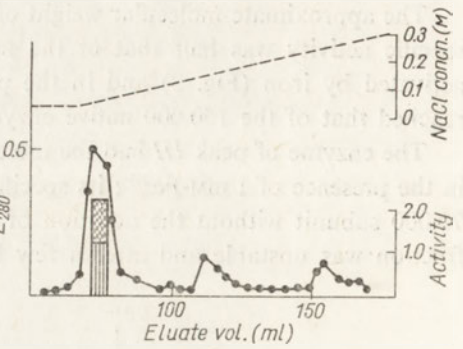

Fig. 7

Fig. 6. DEAE-Sephadex A-50 column chromatography of 70 000 subunit. Protein, 60 mg, of peak *II* from Sephadex G-150 (Fig. 1) was applied to the DEAE-Sephadex A-50 column. Details as in Figs. 4 and 5.

Fig. 7. DEAE-cellulose column chromatography of 70 000 subunit after DEAE-Sephadex A-50 chromatography (Fig. 6). Protein, 30 mg, was applied to DEAE-cellulose column. Details as in Figs. 4 and 5.

Ion-exchange chromatography on DEAE-Sephadex A-50 (Fig. 4) of the enzyme present in Sephadex G-150 peak *I* (native enzyme) gave over twofold purification. The molecular weight was unaltered and the activity was not enhanced by ferrous ion. The active fractions were pooled and applied on DEAE-cellulose column. Active hydroxylase was recovered in 71 - 78 ml of effluent (Fig. 5). Its activity in the presence of 1 mM-Fe^{2+} was nearly doubled, i.e. was characteristic of the 70 000 subunit. However, after molecular sieving on Sephadex G-150, the loss of activity on direct measurement was observed and molecular weight was 40 000.

Ion-exchange chromatography on DEAE-Sephadex A-50 of the 70 000 subunit (peak *II*) gave about threefold purification without affecting molecular weight. The response of activity to Fe^{2+} ion was typically doubled (Fig. 6). After chromatography on DEAE-cellulose (Fig. 7) susceptibility to ferrous ion was the same. Sephadex G-150 gel filtration resulted in disaggregation into inactive 40 000 molecular weight subunits.

Sedimentation and electrophoresis

Preparations of the 150 000 enzyme and 70 000 subunit from DEAE-Sephadex A-50 (step 4) were subjected to sedimentation in the analytical ultracentrifuge (Fig. 8). The proteins sedimented as single homogeneous peaks, and $s_{20,w}$ values for the native enzyme and the subunit were 7.1 and 3.4, respectively. On polyacrylamide gel electrophoresis the preparations were heterogeneous (Fig. 9). The 150 000 enzyme separated into four protein bands, two of which (*A* and *B*) contained the hydroxylase activity. The 70 000 subunit gave on electrophoresis five protein bands; three of them (*C*, *D*, *E*) were found to be active in the presence of Fe^{2+}.

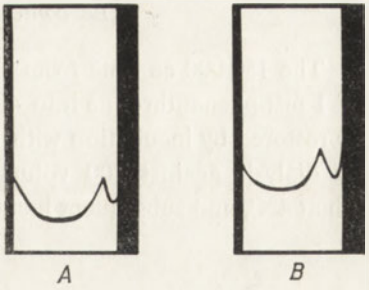

Fig. 8. Ultracentrifuge schlieren patterns of 150 000 hydroxylase and 70 000 subunit from DEAE-Sephadex A-50. The pictures were taken at 32 min after reaching 59 780 rev./min. Sedimentation at 20°C from right to left. *A*, 2.3 mg protein of peak *I*; *B*, 2.6 mg protein of peak *II* in 0.01 M-Na,K-phosphate buffer, pH 7.3.

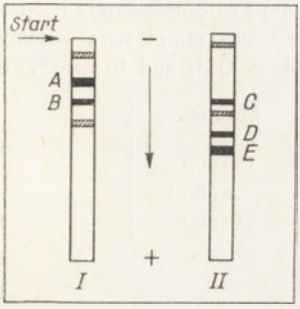

Fig. 9

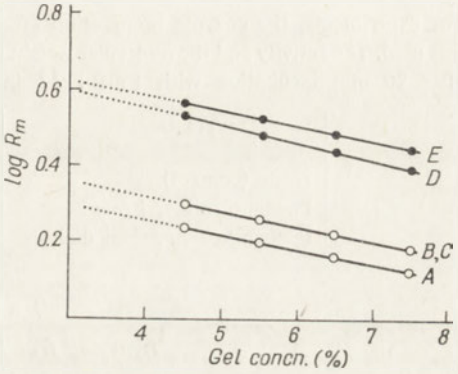

Fig. 10

Fig. 9. Electrophoresis of *I*, pHPP hydroxylase and *II*, 70 000 subunit after DEAE-Sephadex A-50 chromatography. Protein, 15 µg, was layered on the top of the spacer gel and electrophoresis was run for 60 min at 3 mA/gel, pH 9.5. Amido Black was used to stain the protein bands. For activity measurements the gel was sliced into 2 mm discs and the enzyme was eluted with 0.3 ml of 0.01 M-Na,K-phosphate buffer, pH 7.3, for 1 h at 0°C. *A* - *E*, Active bands (determined in the presence of ferrous ion).

Fig. 10. Molecular size determination of pHPP hydroxylase and 70 000 subunit by electrophoresis on polyacrylamide gels of different concentration, according to Hedrick & Smith (1968). The electrophoresis was performed as described for Fig. 9. Migration distance is expressed as log of mobility relative to Amido Black. Bands enzymically active in the presence of 1 mM-Fe^{2+}: *A*, *B*, obtained from 150 000 enzyme, and *C*, *D*, *E*, from 70 000 subunit.

The plot of log mobility relative to Amido Black *versus* polyacrylamide gel concentration according to Hedrick & Smith (1968) gave for bands *A* and *B* two parallel lines (Fig. 10), indicating the same size of protein molecules but different charge. The active bands from 70 000 subunit differ not only in charge but also in size, as indicated by non-parallel lines. The protein band *C* of the same mobility as band *B* may correspond to 70 000 subunit. Proteins *D* and *E*, probably the disaggregation products of 70 000 subunit, are not only charge but also size isomers. The enzyme was not protected from disaggregation by the addition to the gel of substrate or ferrous ion. The number of bands was not increased by 20 min preincubation of enzyme with 1 mM-phenanthroline at 37°C.

The content of iron and free thiol groups

The 150 000 enzyme from DEAE-Sephadex A-50 disaggregated in the presence of 1 mM-phenanthroline into 40 000 subunits and lost its activity, which could not be restored by incubation with 1 mM-Fe^{2+}. The restoration of activity was achieved by dialysis against 100 volumes of 0.01 M-Na,K-phosphate buffer, pH 7.3, for 6 h at 4°C, and subsequent incubation with 1 mM-Fe^{2+} for 20 min at 37°C (Table 2).

Table 2

Reversible effect of o-phenanthroline on activity and molecular weight of pHPP hydroxylase

For experiments, the enzyme preparation after DEAE-Sephadex A-50 chromatography (step 5) was used. The activity and the molecular weight in the phenanthroline-treated sample were measured after 20 min incubation with 1 mM-Fe^{2+} in the presence of 0.4 mM-GSH and 10 mM-DCPP.

Treatment	Specific activity	Mol. wt.
None (control)	0.97	150 000
Phenanthroline, 1 mM	0.00	40 000
Phenanthroline, then dialysis	0.86	150 000

Table 3

Iron and free SH group content

For determination of iron content 40 mg of protein was used. Free SH groups were determined in a sample of 0.25 mg of protein, directly or after 30 min incubation with 0.5% sodium dodecyl sulphate. For details see Methods.

Stage of preparation		Molecular weight	Iron (nmol/mg of protein)	SH (nmol/mg of protein)	
				directly	after SDS treatment
5	DEAE-Sephadex	150 000	13.0	11.7	24
		70 000	12.0	11.4	30
6	DEAE-cellulose	40 000	2.5	27.5	—

Table 4

Effect of sodium dodecyl sulphate on relative activity of the native enzyme and 70 000 subunit after DEAE-Sephadex A-50 chromatography

The activity was determined in the presence of 1 mM-Fe^{2+}, 0.4 mM-GSH, and 10 mM-DCPP and expressed as percentage of the activity without SDS.

SDS added (%)	Activity (%)	
	150 000 enzyme	70 000 subunit
None, control	100	100
0.05	20	40
0.10	0	15
0.20	0	0

Determination of the iron content showed that on DEAE-cellulose and subsequent Sephadex G-150 gel filtration the 150 000 enzyme and 70 000 subunit lost 80% of iron (Table 3) disaggregating into 40 000 subunits. Simultaneously the number of free SH groups doubled and was the same as after 0.5% SDS treatment. The SDS treatment not only raised the number of free thiol groups but also decreased the enzyme activity (Table 4).

Effect of iron and reducing compounds on aggregation and disaggregation of 70 000 subunit

The activity of 70 000 subunit was assayed in the presence or absence of reducing compounds and ferrous ion. After incubation the molecular weight of the protein was determined in a parallel sample by Sephadex G-150 gel filtration (Table 5). It should be noted that in previous experiments molecular weight was determined without the addition of ferrous ion or reducing compounds.

Table 5

The effect of ferrous ion and reducing agents on aggregation and disaggregation of 70 000 subunit

The incubation mixture contained 4 mg of protein (70 000 subunit after DEAE-Sephadex A-50 chromatography), 1.6 μmol of pHPP, 0.01 M-Na,K-phosphate buffer, pH 7.3, to a final volume of 2.6 ml and where indicated: 0.4 mM-GSH, DTT, DTNB, 1 mM-Fe^{2+} and 10 mM-DCPP. After 20 min at 37°C, the enzyme activity was determined. A parallel sample after incubation was applied to Sephadex G-150 column for determination of molecular weight (Fig. 2). The results are expressed as percentage of enzyme recovered in the fraction of indicated molecular weight, and as percentage of the full activity.

Addition	Molecular weight distribution (%)			Activity (%)
	150 000	70 000	40 000	
None	0	100	0	45
GSH, DCPP, Fe^{2+}	100	0	0	100
DTT, DCPP, Fe^{2+}	100	0	0	100
GSH, DCPP	25	75	0	56
GSH	45	30	25	52
GSH, Fe^{2+}	60	40	0	61
DCPP	25	50	25	51
Fe^{2+}	55	45	0	59
DTT	45	30	25	54
DTNB	0	100	0	48

Full aggregation of 70 000 subunits to the 150 000 enzyme and restoration of its activity was observed in the presence of ferrous ion, GSH and DCPP together. Partial aggregation was achieved by two reducing agents or by ferrous ion alone. When only one of the reducing agents was present, aggregation to 150 000 and disaggregation to 40 000 subunits was observed. The SH-complexing Ellman's reagent (DTNB) had no effect on enzyme activity or molecular weight.

DISCUSSION

The presented results indicate that *p*-hydroxyphenylpyruvate hydroxylase from rabbit liver, with a molecular weight of about 150 000, is composed of four inactive 40 000 mol. wt. subunits. These subunits are size and charge isomers as demonstrated by their mobility on polyacrylamide gels of different concentration.

The tetrameric structure of the enzyme is maintained by ferrous ions which are protected from oxidation by SH groups. Partial oxidation of ferrous ion leads to disaggregation of the enzyme into two dimers of 70 000 mol. wt. with unchanged percentage content of iron but with diminished activity. Removal of iron leads to disaggregation into monomeric subunits. The addition of Fe^{2+} and SH-containing compounds restores full activity of the dimeric units connected with regeneration of native enzyme. Also the inactive, monomeric subunits aggregated into the 150 000 enzyme.

It is well known that on storage at $-20°C$ the hydroxylase loses its activity which may be restored by DCPP (Zannoni & La Du, 1959; Goswami, 1964). From our experiments it appears that in a molecule of the native enzyme not all iron ions are to the same degree susceptible to oxidation and dissociation. The enzyme precipitated with ammonium sulphate and desalted on Sephadex G-50 retained its tetrameric structure but on Sephadex G-150 some molecules underwent dissociation (Fig. 1). The chromatography on DEAE-cellulose caused further oxidation of iron with simultaneous disaggregation of the enzyme into dimeric forms (Fig. 5) which on Sephadex G-150 gel filtration disaggregated into monomeric subunits as a result of almost complete loss of iron. Addition of ferrous ion and SH-containing compounds prevented this disaggregation.

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PODJEDNOSTKOWA BUDOWA HYDROKSYLAZY *p*-HYDROKSYFENYLOPIROGRONIANU Z WĄTROBY KRÓLIKA

Streszczenie

1. Wykazano podjednostkową budowę hydroksylazy *p*-hydroksyfenylopirogronianu. Oznaczony sączeniem molekularnym przybliżony ciężar cząsteczkowy enzymu wynosił 150 000, a stała sedymentacji $s_{20,w}$ 7,1. Ciężar cząsteczkowy podjednostki wynosił 70 000, a $s_{20,w}$ 3,4. Obserwowano również dezagregację do nieaktywnych podjednostek o masie 40 000.

2. Elektroforezą na żelu poliakrylamidowym wykazano różnice w ładunku i wielkości podjednostek o m.cz. 40 000.

3. Wyniki doświadczeń nad wpływem Fe^{2+} i związków zawierających SH wskazują na udział dwuwartościowego żelaza w tetramerycznej budowie enzymu i na rolę SH w utrzymaniu żelaza w stanie zredukowanym. Usunięcie żelaza z cząsteczki powoduje dezagregację oraz utratę aktywności hydroksylazy. Dezagregację można odwrócić przez dodanie Fe^{2+}, co związane jest z odzyskaniem aktywności.

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PREPARATION AND PROPERTIES OF POLY 2-AMINOPURINE RIBOTIDYLIC ACID

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1. A new polynucleotide phosphorylase substrate, 2-aminopurine riboside 5′-pyrophosphate, has been prepared by dechlorination of 2-amino-6-chloropurine riboside, enzymic phosphorylation of the resulting 2-aminopurine riboside with wheat shoot phosphotransferase, and chemical phosphorylation of the 5′-phosphate *via* the morpholidate. 2. Polymerization of the substrate to give poly 2-aminopurine ribotidylic acid proceeded with the *E coli* enzyme in the presence of Mn^{2+}, but not Mg^{2+}, cations. 3. The resulting homopolymer, poly(2APR), was markedly heterogeneous. Although hydrolysis to mononucleotides was accompanied by 15-20% hyperchromicity, indicative of appreciable base stacking, the polymer exhibited virtually no temperature hyperchromicity. Poly(2APR) also did not form a protonated helical structure in acid medium. 4. Poly(2APR) readily formed helical structures with poly(rU). The stoichiometry of this reaction was in the neighbourhood of 1:1 for samples with values of $s_{20,w}$ about 5, but this ratio was closer to 2:1 for samples with low sedimentation constants. The complexes with poly(rU) exhibited relatively sharp transition profiles, typical for helical complexes. Furthermore, the CD spectra of the complexes differed appreciably from those for the sum of the components; in particular the principal long-wavelength band was negative. 5. The homopolymer showed no evidence of complex formation with either poly(rC) or poly(rI), nor with hydroxylamine or methoxyamine modified homopolymers of poly(rC). 6. The fluorescence properties of the homopolymer and its complex with poly(rU) have been examined and compared with those previously reported for alternating copolymers of 2APR and rU. 7. The overall results are also discussed in relation to hydroxylamine-induced mutagenesis.

One of the most potent mutagens amongst the known base analogues is 2-aminopurine (2AP)[1]. Its mutagenic action, which results in the production exclusively of transitions (Koch, 1971), has been ascribed in part to its duality in base-pairing

[1] Non-usual abbreviations employed in this text: 2AP, 2-aminopurine; 2APR, 2-aminopurine riboside; 2APdR, 2-aminopurine deoxyriboside; poly(2APR), poly 2-aminopurine ribotidylic acid; poly(5MedC), poly 5-methylcytidylic acid; poly(N^4-hydroxyC), poly N^4-hydroxycytidylic acid; poly(N^4-methoxyC), poly N^4-methoxycytidylic acid.

properties, based on the presumed existence of the imino along with the amino form (Freese, 1959). The analogue may undergo incorporation into both the DNA and RNA of *E. coli*, the DNA of phage T_4, etc. but the limited degree of incorporation has hitherto foiled attempts to determine directly which base(s) it substitutes for (Wacker *et al.*, 1960; Gottschling & Freese, 1961).

Rogan & Bessman (1970) recently investigated enzyme specificity towards several 2-aminopurine derivatives, and found that the analogue may be recognized as G or A, depending on the reaction involved. Formation of the 5′-pyrophosphate from 2-aminopurine ribose-5′-phosphate was effected by guanylate, but not adenylate, kinase. By contrast, the triphosphate of 2APdR could substitute for dATP, but not dGTP, in the DNA polymerase system. A similar situation appears to prevail with the RNA polymerase system: with poly d(A-T) as template, a series of alternating ribose copolymers of the type poly r(2AP-U) were readily obtained (Ward *et al.*, 1969).

There is one report extant on the *in vitro* messenger activity of poly(2APR), indicating that the 2AP residues code more like adenine than guanine (Wacker *et al.*, 1966). However, no data were presented with regard to the properties of the poly(2APR) used as messenger.

Our interest in poly(2APR) stemmed largely from investigations on the mechanism of mutagenesis with hydroxylamine and its reaction with cytosine residues in nucleic acids. Poly(2APR) appeared to be a good model homopolymer for possible base pairing with poly(N^4-hydroxyC), irrespective whether the residues of the latter exist in the amino and/or imino forms, a problem which is still to be resolved unequivocally (Kochetkov *et al.*, 1967; Brown *et al.*, 1968; Janion, 1972).

MATERIALS AND METHODS

2-Amino-6-chloropurine riboside was obtained from Waldhof Zentralstoffabrik (Mannheim, G.F.R.), and *p*-nitrophenylphosphate from Koch-Light (Colnbrook, Bucks., England). Poly(rU), poly(rA), poly(rC) and poly(rI) were either products of Miles Laboratories (Elkhart, Indiana, U.S.A.) or/and were prepared in this laboratory by standard procedures.

Poly(N^4-hydroxyC) and poly(N^4-methoxyC) were prepared as elsewhere described (Janion & Shugar, 1968).

Escherichia coli polynucleotide phosphorylase (spec. act. 74.5, 6.25 mg of protein/ml) was a gift from Dr. Marianne Grunberg-Manago (Williams & Grunberg-Manago, 1964). Wheat shoot nucleoside phosphotransferase was prepared as described by Barner & Cohen (1959) and the crude extract used as such. Pancreatic ribonuclease was a crystalline preparation from Worthington (Freehold, N.J., U.S.A.).

Spectral measurements and thermal profiles were run on a Unicam SP-500 instrument fitted with a specially constructed copper-jacketted cuvette compartment through which was circulated aqueous glycol from a Hoeppler ultrathermostat. A thermistor in a dummy cuvette was employed for temperature measurements.

Formation of complexes was followed by measurements of hypochromicity at the appropriate wavelength(s) on mixing the two components in varying proportions, as previously described (Janion & Shugar, 1969).

All pH measurements made use of a Radiometer PHM22 instrument fitted with a semi-micro glass electrode.

CD spectra were run on solutions with optical densities of about 1.5, the instrument employed being a JASCO ORD/UV-5 spectropolarimeter with circular dichroism attachment. Sedimentation constants of polymers, in 0.01 M-phosphate buffer, pH 7.2, and 0.5 M-NaCl were obtained with a Beckman Model E instrument fitted with ultraviolet optics.

Preparation of 2-aminopurine riboside 5'-pyrophosphate

The starting product was 2-amino-6-chloropurine riboside and the procedure embraced the following steps:

2-Aminopurine riboside: 2-amino-6-chloropurine riboside, in 100 mg portions in 50 ml water, with addition of 5g Zn, was heated at 90°C for 30 - 60 min, until an aliquot appropriately diluted exhibited a 38 - 40% decrease in UV absorption at 305 nm on changing the pH from 7 to 2. The solution was then filtered, concentrated under reduced pressure, deposited on 20 - 24 cm sheets of Whatman 3*MM* paper, and developed with 7 : 3 (v/v) ethanol : 1 M-ammonium acetate. The 2-aminopurine riboside bands (R_F 0.69) were eluted with water and the combined eluates brought to dryness under reduced pressure. Occasionally a faint band of starting material (R_F 0.80), and faint bands of other unidentified products, were visible, but these did not interfere with the isolation of the dechlorination product in yields averaging about 70%. This was used as such in the next step.

2-Aminopurine riboside 5'-phosphate: This was obtained by enzymic phosphorylation of 2-aminopurine riboside with wheat shoot nucleoside phosphotransferase using *p*-nitrophenylphosphate as the donor. The reaction mixture, total volume 12 ml, included 4 ml 0.2 M-acetate buffer, pH 4, about 0.25 mM-2APR, 3.5 mM (1.2 mg) *p*-nitrophenylphosphate, and 5 ml (6.7 mg protein) of the enzyme solution. Incubation was overnight at 37°C. The course of the reaction was followed chromatographically, using Whatman no. 1 paper and development with 7 : 3 ethanol: :1 M-ammonium acetate. R_F values were: 2-aminopurine riboside 5'-phosphate, 0.18; 2-aminopurine riboside, 0.69; *p*-nitrophenylphosphate, 0.48; *p*-nitrophenol, 0.95. The reaction yield, calculated by elution and spectrophotometry of the spots, was 60% after about 18 h incubation. The nucleotide, and unreacted nucleoside, were recovered on a preparative scale by chromatography on Whatman 3*MM* paper.

2-Aminopurine riboside 5'-pyrophosphate: The ammonium salt of the 2-aminopurine riboside 5'-phosphate was first converted to the free acid by addition of Dowex 50WX8 (200/400 mesh) to an aqueous solution. The resin was removed by filtration and the filtrate brought to dryness. Conversion of the free acid to the morpholidate and then to the 5'-pyrophosphate followed essentially the procedure of Moffatt & Khorana (1961), except the isolation of the final product.

Following completion of the pyrophosphorylation step, the reaction mixture was brought to dryness, freed from pyridine, dissolved in water and applied to a 25×1.5 cm column of DEAE-cellulose (HCO_3^- form). The products were eluted with a linear gradient formed from 1.3 litre water and 1.3 litre 0.3 M-triethylammonium bicarbonate, pH 7.5. Fractions of 15 ml were collected, the elution pattern being as follows (figures in parentheses indicate molarity of eluent): 2-aminopurine riboside and the morpholidate of its 5′-phosphate (0.02 - 0.03), inorganic phosphate (0.02-0.042), 2-aminopurine riboside 5′-phosphate (0.065 - 0.075), 2-aminopurine riboside 5′-pyrophosphate (0.10 - 0.105).

The pooled fractions containing the 5′-pyrophosphate were brought to dryness under reduced pressure and freed from excess triethylammonium bicarbonate by several evaporations from methanol. The triethylammonium salt was either used as such, or was converted to the sodium salt by passage through a column of Dowex 50W (50X2-200), Na^+ form. The overall yield of the pyrophosphate, relative to the monophosphate, was 24%.

Polymerization of 2-aminopurine riboside 5′-pyrophosphate

Initial attempts at polymerization with Mg^{2+}, under a wide variety of conditions, were unsuccessful. Polymer formation was noted, using paper chromatography, only when Mg^{2+} was replaced by Mn^{2+}. The apparent Mn^{2+} dependence of enzyme activity towards this substrate is similar to that previously reported for polymerization of 2′-*O*-methylcytidine-5′-pyrophosphate (Żmudzka *et al.*, 1969b; Janion *et al.*, 1970).

Optimal conditions for polymerization were as follows (total volume 1 ml): 3 μM-substrate, sodium salt; 40 μl 0.01 M-Na-EDTA; 300 μl 0.5 M-tris buffer, pH 8.5; 30 μl 0.1 M-$MnCl_2$; 100 μl 0.01 M-NaN_3 (to prevent bacterial contamination); 50 μl enzyme. Incubation was for 48 h at 37°C. The reaction mixture was then deproteinized twice with neutral phenol, the aqueous phase extracted with ether to remove phenol, and nitrogen bubbled through the solution to remove ether. The polymer solution was then dialysed successively against 0.1 M-NaCl plus 0.01 M-EDTA, 0.01 M-NaCl plus 0.001 M-EDTA, redistilled water, and then lyophilized.

The yield of the polymerization reaction, based on measurements of disappearance of substrate, was of the order of 30%. The product obtained by the foregoing procedure exhibited a relatively low $s_{20,w} \sim 1.6$, with a heterogeneous sedimentation pattern. Attempts to obtain products with higher sedimentation constants under these conditions were unsuccessful.

When the sodium salt of the substrate was replaced by the triethylammonium salt, optimal yields of polymer product were obtained at 3-fold higher concentrations of substrate and Mn^{2+} cations. Following 24 h incubation, the resulting poly-(2APR) exhibited an $s_{20,w}$ of about 3. Prolongation of the incubation period to 48 h led to an increase in $s_{20,w}$ to about 5, but the product now exhibited marked heterogeneity.

RESULTS

Properties of poly(2APR)

The UV absorption spectrum of poly(2APR) at neutral or alkaline pH is shown in Fig. 1. The same figure exhibits the spectrum following alkaline hydrolysis to mononucleotides, which shows that the accompanying appreciable hyperchromicity varies from 15 - 20% at 240 nm and 305 nm for different polymer preparations. Using the value of Fox *et al.* (1958) for the molar extinction coefficient of 2-aminopurine riboside at λ_{max} 305 nm, 6.9×10^3, it follows that the molar extinctions of poly-(2APR) preparations at 305 nm vary from 5.7 to 6.0×10^3.

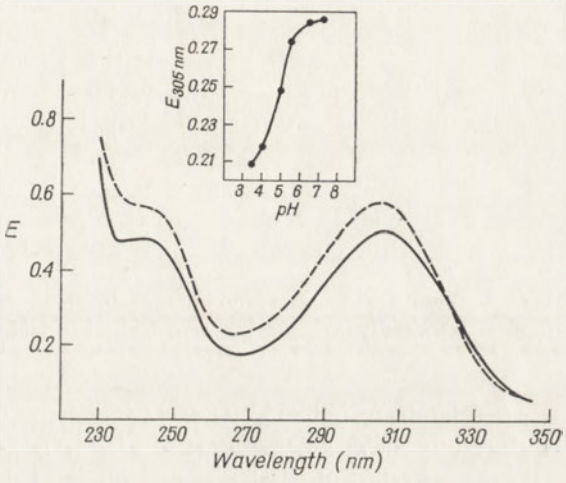


Fig. 1. UV absorption spectrum of poly(2APR) at neutral or alkaline pH (——), and following alkaline hydrolysis to mononucleotides (- - -). Insert shows pH-dependence of absorption at λ_{max}, 305 nm.

The hyperchromicity accompanying hydrolysis to mononucleotides, as well as the attendant low (but clearly defined) hypochromicity at wavelengths to the red of 325 nm, testifies to the existence of poly(2APR) in some structured form, probably as a result of base stacking. It is consequently of interest that heating of poly(2APR) in 0.01 M-phosphate buffer, pH 7.2, in the presence of up to 0.5 M-Na^+, at 90°C, led to the appearance of only 2% hyperchromicity at 305 nm. Actually, if the volume expansion of the solution is taken into account, the temperature hyperchromicity is 4 - 5%, which is a relatively low value.

Additional evidence for appreciable base stacking in poly(2APR) is furnished by a study of its emission properties, as well as by its CD spectrum. Like adenosine and AMP (Green & Mahler, 1970), 2APR shows practically no Cotton effect over the wavelength range 230 - 330 nm. By contrast, poly(2APR) exhibits a characteristic CD spectrum (cf. curve *b* in Fig. 6) with a positive band centred at about 310 nm. The absence of a cross-over point with a negative band at shorter wavelengths is rather odd, but was typical of all preparations examined.

Since 2APR, like adenosine, protonates in acid medium, with a p*K* of 3.4 (Fox *et al.*, 1958), it became of interest to determine whether poly(2APR) is capable of forming an acid twin-helical structure like poly(rA). Whereas adenosine is known

to protonate on the ring N_1 nitrogen, the site of protonation in 2APR has not been established. Ikeda *et al.* (1970), on the basis of a comparison of the infrared absorption spectra of adenosine, 2-amino-N^6-methyladenosine, the homopolymers of both of these, and 1-hydroxyethyladenine, concluded that a band at 1665 cm^{-1} is characteristic for protonation of the N_1 ring nitrogen. From Fig. 2 it will be noted that the same band, absent in the neutral form of 2APR, appears in the protonated form; on this basis, we conclude that the site of protonation is likewise the ring N_1.

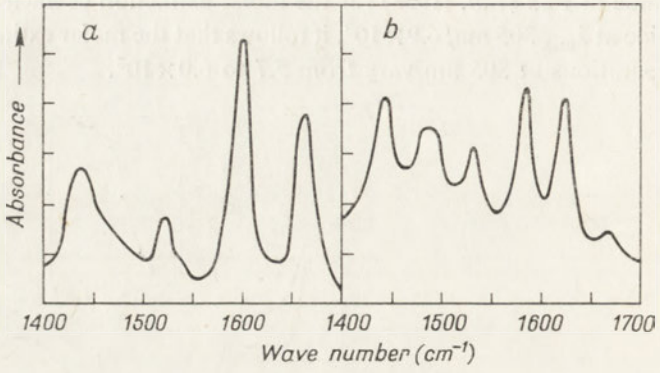


Fig. 2. Infrared absorption spectrum of 0.1 M-2-aminopurine riboside in D_2O, (*a*) cationic form in 0.1 N-HCl; (*b*) neutral form in phosphate buffer, pD 5.9. Path length of cuvette 50 μ.

A solution of poly(2APR) was titrated spectrally from neutral to acid medium, with results shown in the insert to Fig. 1. The lower attainable limit for titration was pH 3.5, since the polymer precipitated below this pH. However, the titration curve not only demonstrates the absence of any abrupt transition such as is observed with poly(rA) in acid medium, but approximates to a normal titration curve, from which it appears that the 2APR residues in the polymer protonate with an average "pK" of about 4.8. When compared with the pK of 3.4 for the monomer, this further

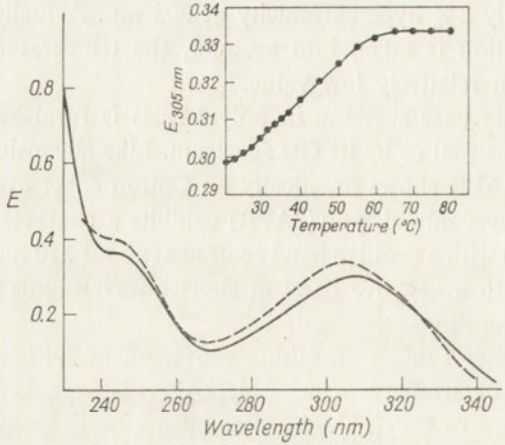


Fig. 3. UV absorption spectrum of poly-(2APR) in 0.02 M-acetate buffer, pH 4.4, at 20°C (——) and at 80°C (- - -). Insert shows temperature-dependence of absorption at 305 nm at pH 4.4.

testifies to appreciable base stacking in poly(2APR). The absence of any transition to a protonated helical form is confirmed by the absorption spectrum of the polymer at pH 4.4, i.e. in the region of the pK of the 2APR residues in the polymer. The spectrum resembles the protonated form of 2APR and, on heating to 80°C, is modified so that it approximates to the spectrum of the neutral form (Fig. 3), pointing to temperature-induced deprotonation. The non-cooperative nature of this modification as a function of temperature can be seen in the insert to Fig. 3.

Complexing ability of poly(2APR)

The ability of poly(2APR) to form complexes with other potentially complementary polynucleotides was examined in some detail, principally by searching for changes in UV absorption differing from additivity, over entire wavelength range of absorption, following mixing of the two components in proportions varying from 1 : 2 to 2 : 1.

No hypochromicity (or hyperchromicity) could be detected in mixtures of poly(2APR) and poly(rC). Watson-Crick base-pairing is excluded in this case, but model-building studies show that base-pairing is possible in which the 2APR amino group bonds to the cytosine N_3 ring nitrogen and the cytosine amino group to the ring N_1 of 2APR. With such a pairing scheme, the relative orientation of the glycosidic bonds is not incompatible. Nonetheless the results pointed to the absence of any complex formation. The results with poly(rI) were equally negative, although here also formation of two bonds per base pair is possible.

No observable interaction was found with either poly(N^4-hydroxyC) or poly(N^4-methoxyC). There is still some disagreement as to whether the modified cytosine residues in the latter polymers are in the amino or imino form (or possibly a mixture of the two). If the imino form prevailed, one might have anticipated complex formation, as with poly(rU). However, it is conceivable that the stability of such complexes might be too far below room temperature to be detectable.

Complex formation of poly(2APR) with poly(rU)

Mixing of solutions of poly(2APR) and poly(rU) led to the almost instantaneous appearance of hypochromicity and, at longer wavelengths, hyperchromicity, testifying to complex formation (Fig. 4). However, the stoichiometry of this reaction was found to be dependent on the chain length of the poly(2APR) preparations, as shown by the typical experimental mixing curves in Fig. 5.

With preparations of poly(2APR) for which the $s_{20,w}$ value was 1.5, maximum hypochromicity was observed at 2APR : rU ratios of 2 : 1. With poly(2APR) exhibiting an $s_{20,w}$ of 2.9, the ratio decreased to 1.5, and this decreased further to 1.3 for the poly(2APR) sample showing an s value of about 5. It should, however, be recalled that even this latter preparation was quite heterogeneous (see above), from which it appears reasonable to infer that the complexing ratio would extrapolate to 1 : 1 with a homogeneous sample exhibiting an s value above 5. It should be

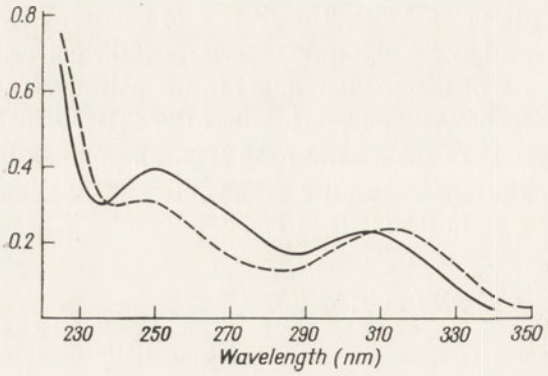


Fig. 4. UV absorption spectrum, in 0.01 M-phosphate buffer, pH 7.2, and 0.5 M-Na^+, of a 1.5 : 1 mixture of poly(2APR) and poly(rU) (- - -), as compared to the arithmetic sum of the absorptions of the two components (——). Note the pronounced hyperchromicity of the mixture to the violet of 240 nm and to the red of 310 nm.

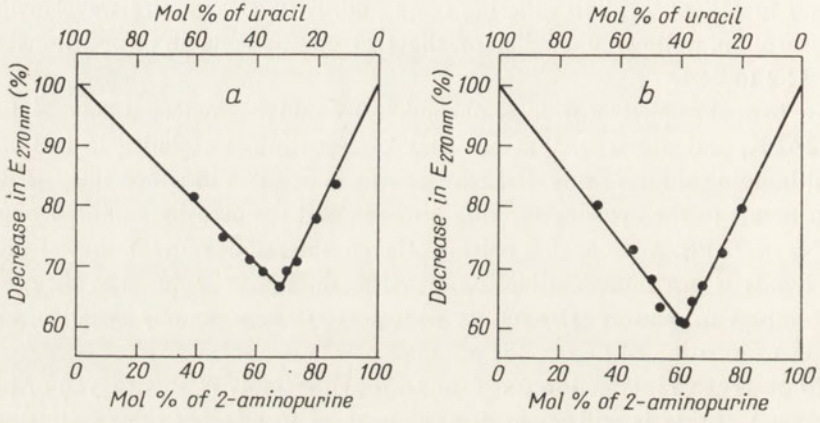


Fig. 5. Mixing curves of poly(2APR) with poly(rU) in 0.01 M-phosphate buffer, pH 7.2, and 0.1 M-NaCl, as followed by hypochromicity at 270 nm: (*a*) poly(2APR) sample with $s_{20,w}$ 1.5; (*b*) poly(2APR) sample with $s_{20,w} \sim 2.7$.

noted that base-pairing between 2APR and U residues is possible only in the ratio 1 : 1 (see Discussion).

We now present additional evidence for complex formation between poly(2APR) and poly(rU), as well as some of the properties of these complexes.

Circular dichroism spectra. Striking evidence for formation of a specific complex between the two homopolymers is furnished by a comparison of the CD spectrum for the mixture relative to that of each of the components (Fig. 6). Note in particular the change in sign of the positive band at about 310 nm, originating in the homopolymer components uniquely from poly(2APR), to one with negative sign and increased amplitude in the complex. The monomer 2APR exhibited no detectable optical activity under these conditions.

Fluorescence of poly(2APR) and its complex with poly(rU). In agreement with the known spectral emission properties of 2APR residues (Ward *et al.*, 1969), poly(2APR) in 0.01 M-phosphate buffer, pH 7.2, and 0.5 M-NaCl exhibited an emission spectrum with a maximum at 370 nm and a quantum yield for fluorescence

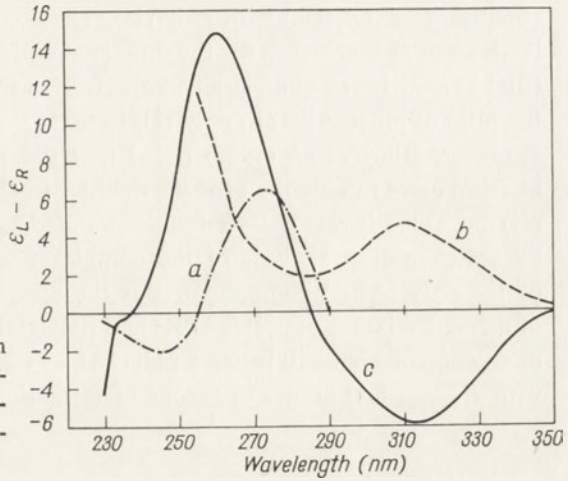


Fig. 6. Circular dichroism spectra, in 0.01 M-phosphate buffer, pH 7.2, and 0.1 M-NaCl, at room temperature, of: (*a*) poly(rU), (*b*) poly(2APR), (*c*) poly(2APR)·poly(rU) in the ratio 1.8 : 1.

approximately 2% that for the monomer. The excitation maximum was located at about 303 nm. The low quantum yield for emission of 2APR residues following incorporation into a polynucleotide chain is quantitatively in accord with the findings of Ward *et al.* (1969) for perfectly alternating copolymers of 2APR with other base residues. Presumably fluorescence quenching is due to interactions resulting from base stacking, and the present results show that such quenching may result from stacking of a 2APR residue not only with another base, as in alternating copolymers of 2APR (Ward *et al.*, 1969), but also with adjacent 2APR residues. It would consequently be of interest to examine the effects of base stacking of 2APR residues, or better still 2-amino-9-ethylpurine, in aqueous medium on the emission properties. It should, furthermore, be noted that the decreased quantum yield for emission of 2APR residues in alternating copolymers was ascribed (Ward *et al.*, 1969) to residual emission from the residues at the chain termini. In any event the dramatic decrease in quantum yield of 2APR residues in poly(2APR) further supports the arguments presented above for some structured form of the latter and similar to that ascribed to base stacking in such polymers as poly(rA).

Mixing of poly(2APR) with poly(rU) led to a further reduction in fluorescence quantum yield of about 50%, but with the emission maximum unchanged relative to that for uncomplexed poly(2APR), i.e. 370 nm. The specificity of this additional quenching was testified to by the fact that it was unaltered on addition of an excess of poly(rU). Finally, treatment of the complex with pancreatic RNase at temperatures above the T_m value (see below), leading to hydrolysis of the poly(rU) component, regenerated the initial fluorescence intensity of poly(2APR).

Temperature transition profiles. Heating of mixtures of poly(2APR) with poly(rU) demonstrated the existence of relatively sharp transition profiles (Fig. 7), typical of helix-coil transitions. Note that the T_m value for the transition increases with the Na^+ concentration, as normally observed for helical polyribonucleotide complexes, and that in the neighbourhood of 1 M-Na^+ the breadth of the transition is appreciably reduced. The T_m values are somewhat lower than those for the corresponding 1 : 1

complexes of poly(rA) with poly(rU). The increase of T_m with Na^+ concentration is also not as marked as for the double-stranded complex of poly(rA) with poly(rU) (Blake *et al.*, 1967), but a strict comparison of the two systems is not warranted since the ratio of poly(2APR) to poly(rU) in these experiments is somewhat higher than 1. However, Riley *et al.* (1966) noted that the T_m values for triple-stranded helices increase more rapidly than for the double-stranded ones; in this respect poly(2APR-rU) more closely resembles a double-stranded helix. Attempts to resolve this question by sedimentation analysis of the complexes were hampered by the heterogeneity of the poly(2APR) component. But the appearance in the sedimentation pattern of a 1.8 : 1 mixture of poly(2APR) and poly(rU) of a major component with a maximum $s_{20,w}$ of about 10 [using a poly(2APR) sample with $s_{20,w} \sim 2.7$ and a poly(rU) with $s_{20,w} = 6.2$] argues in favour of formation of a 1 :1 complex.

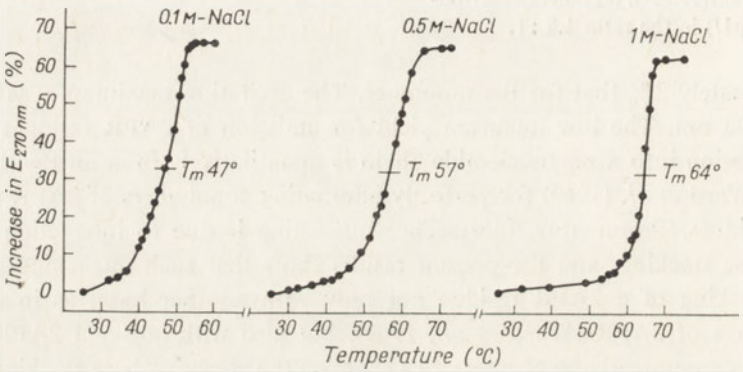


Fig. 7. Thermal transition profiles in 0.01 M-phosphate buffer, pH 7.2 , and various Na^+ concentrations as indicated, for poly(2APR)·poly(rU), 1.5 : 1; T_m values are indicated beside each profile.

DISCUSSION

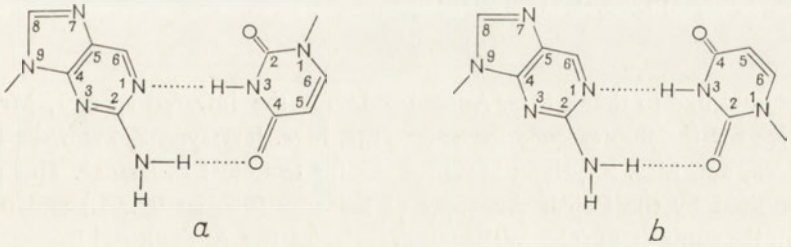
The apparent Mn^{2+} dependence of *E. coli* polynucleotide phosphorylase for polymerization of the 2-aminopurine riboside 5′-pyrophosphate is in line with the previously observed Mn^{2+} dependence of the *E. coli* and *M. lysodeikticus* enzymes with respect to such substrates as 2′-*O*-methylcytidine-5′-pyrophosphate (Janion *et al.*, 1970; Żmudzka *et al.*, 1969b) and 2′-*O*-methyluridine-5′-pyrophosphate (Janion, unpublished observations; Żmudzka & Shugar, 1970). This Mn^{2+} requirement, as compared to the normally observed Mg^{2+} dependence, has since been reported by several other observers (Chou & Singer, 1971; Torrence & Witkop, 1972; Hobbs *et al.*, 1972).

It is, however, rather difficult to conclude whether the observed Mn^{2+} dependence is absolute, largely because of the varying degrees of purity of different polynucleotide phosphorylase preparations, even those from the same source. It should be recalled that Thang *et al.* (1965) had earlier observed that the polymerization of GDP by polynucleotide phosphorylase proceeds more readily, but not exclusively, in the presence of Mn^{2+} as compared to Mg^{2+} (see also Babinet *et al.*, 1965).

As regards the homopolymer poly(2APR), and notwithstanding the heterogeneity of the preparations obtained, several facts testify to its existence in some structural form probably involving extensive base stacking. These are: (a) the appreciable hyperchromicity on hydrolysis to mononucleotides, (b) the spectral titration results in acid medium, (c) the dramatic fluorescence quenching relative to the free monomers, (d) the CD spectrum of the homopolymer as compared to that of the monomer. The complete absence of temperature hyperchromicity is rather unexpected, but by no means unusual; similar absence of temperature hyperchromicity has been noted for poly(dC) and poly(5MedC) (Żmudzka *et al.*, 1969a), the circular dichroism spectra of which testify to the presence of appreciable base stacking (Adler *et al.*, 1968). Finally, assuming the validity of the results of Wacker *et al.* (1966) for the messenger activity of poly(2APR), which is of the same order of magnitude as that for poly(rA), these point to the absence of appreciable, if any, helical structure.

Considering now the complex(es) formed between poly(2APR) and poly(rU), the marked tendency towards a 1 :1 ratio of 2APR : rU as the sedimentation constant of the poly(2APR) component increases, points to the formation of a 1 :1 helical complex. The relatively sharp, single-stage, melting profiles (Fig. 7) are consistent with such an interpretation. The observed deviations from unity of the 2APR : rU ratio would then be due to the weak interaction of oligonucleotides of 2APR or their elimination from the helical structure by the longer chains. Fresco (1963) initially reported that oligo(rG) interacted with poly(rC) in the ratio of 2 :1. It was subsequently shown by Pochon & Michelson (1965) that poly(rG) formed a 1 :1 helical complex with poly(rC). However, when the poly(rG) component was fragmented to shorter chains, the apparent interaction ratio approximated 2 : 1.

Assuming, as appears reasonable, that 2APR is in the amino form, two types of base-pairing are possible, as illustrated in Scheme 1. Either of these appears



Scheme 1. The possible base-pairing between 2-aminopurine and uracil residue.

feasible when considered in terms of the relative orientations of the phosphodiester chains. Mixed 1 :1 crystals of 2-amino-9-ethylpurine and 1-methyl-5-bromouracil exhibit uniquely base-pairing such as that illustrated in Scheme 1b. This does not necessarily prove that such base-pairing occurs in helical complexes in solution. Particularly relevant in this respect is the long-wavelength negative band in the CD

spectrum of the poly(2APR-rU) complex (Fig. 6). In the case of poly 2-amino-6-methylaminoadenylic acid, which forms a 1 : 1 helical complex with poly(rU), and the CD spectrum of which likewise exhibits a long-wavelength negative band, this has been interpreted as due conceivably to steric hindrance of the N^6 methyl group, leading to deviations from coplanarity of the bases or some modified tilt of the latter in the helix (Ikeda *et al.*, 1970). No such interpretation can be advanced in the case of complexes of poly(2APR) with poly(rU). However, one possibility not considered by the foregoing authors, is the existence of the purine residues in the *syn*, as compared to the *anti*, form, which would be consistent with the observed negative sign of the long-wavelength extremum (Fig. 6).

The failure of poly(N^4-hydroxyC), or poly(N^4-methoxyC), to interact with poly-(2APR) is relevant to the problem of the mechanism of hydroxylamine-induced mutagenesis, which is widely considered to involve base-pair transitions *via* modifications of cytosine residues to N^4-hydroxycytosine (or N^4-methoxycytosine with the use of methoxyamine). If these modified residues exist in the imino form, or at least partially in the imino form, they would be expected to base-pair like uracil. The results presented above demonstrate unequivocally that, although poly(2APR) readily interacts with poly(rU) to form a helical complex, it does not interact with either poly(N^4-hydroxyC) or poly(N^4-methoxyC). These findings are consistent with those of previous investigations, where it was shown that poly(rA) did not interact with poly(N^4-hydroxyC) (Janion & Shugar, 1968) or with copolymers containing various proportions of N^4-hydroxyC residues (Janion & Shugar, 1969). The failure of the hydroxylamine-modified poly(rC) to complex with poly(2APR) cannot, of course, be used as an argument against the existence of the former in the imino form; e.g. it is equally surprising, in view of the ability of poly(2APR) to complex with poly(rU), that it will not interact with poly(rI). The overall results do suggest that the generally accepted mechanism of hydroxylamine-induced mutagenesis may require re-evaluation.

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SYNTEZA I WŁAŚCIWOŚCI KWASU POLI 2-AMINOPURYNORYBOTYDYLOWEGO

Streszczenie

1. 5′-Pirofosforan rybozydu 2-amino puryny, który, jak stwierdzono, jest substratem fosforylazy polinukleotydowej, otrzymano w wyniku: odszczepienia cząsteczki chloru z rybozydu 2-amino-6-chloropuryny, enzymatycznej fosforylacji przy udziale fosfotransferazy siewek pszenicy, a następnie chemicznej fosforylacji 5′-fosforanu rybozydu 2-aminopuryny do 5′-pirofosforanu.

2. Enzym *E. coli* polimeryzuje 5′-pirofosforan 2-aminopuryny w obecności jonów Mn^{2+}, ale nie katalizuje polimeryzacji w obecności jonów Mg^{2+}.

3. Powstający homopolimer (2APR) jest heterogenny. Po hydrolizie do mononukleotydów obserwuje się 15 - 20% hiperchromazję, co wskazuje na znaczne „przyleganie" zasad, brak jest natomiast zmian w absorpcji poli(2APR) przy wzroście temperatury. Poli(2APR) nie tworzy również heliksu w środowisku kwaśnym.

4. Poli(2APR) łatwo tworzy struktury heliksowe z poli(rU). Stechiometria tej reakcji była bliska 1:1 dla preparatów o stałej sedymentacji $s_{20,w}$ około 5, ale stosunek ten zbliżał się do 2:1 przy niższych stałych sedymentacji. Kompleksy z poli(rU) dają względnie ostre profile tranzycji typowe dla kompleksów heliksowych. Widmo dichroizmu kołowego kompleksu również znacznie odbiegało od widma sumy składników.

5. Poli(2APR) nie tworzy kompleksów z poli(rC) czy poli(rI), ani też z homopolimerami poli(rC) zmienionymi w wyniku reakcji z hydroksylaminą lub metoksyaminą.

6. Zbadano fluorescencję poli(2APR) i jego kompleksów z poli(rU) i porównano ją z uprzednio opisaną fluorescencją polimerów 2APR i rU.

7. Wyniki poddano dyskusji w powiązaniu z mutagennym działaniem hydroksylaminy.

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PROTEOLYTIC DEGRADATION OF POLYMYXINS BY THE ENZYMES OF *BACILLUS POLYMYXA*

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1. Extracellular neutral proteases have been isolated from *Bacillus polymyxa* d/13 K-1 producing polymyxin E (collistin) and from *Bacillus polymyxa* ATCC 10401 synthesizing polymyxin D. 2. The enzymes were purified about 50-fold by ammonium sulphate fractionation, lead acetate treatment and Sephadex G-100 chromatography. 3. Both enzymes decompose polymyxin E but are ineffective with polymyxin D. 4. General characteristics of the enzymes and the mode of their action on polymyxins are discussed.

The antibiotic peptides are generally resistant to the action of proteolytic enzymes. Degradation of penicillins, cephalosporins and actinomycins involves non-peptide linkages. Proteolysis of gramicidin S by protease of *Bacillus subtilis* (Yukioka *et al.*, 1966) and of polymyxins by plant and bacterial enzymes (Suzuki *et al.*, 1963; Paulus & Gray, 1964; Ito *et al.*, 1966) are exceptional in this respect.

Polymyxins are decapeptides composed of cyclic heptapeptide with a tripeptide side-chain containing the acyl residue (R) of 6-methyloctanoic acid or 6-methylheptanoic acid:

L-Thr ← L-DABA ← L-DABA ← **Y** ← **X** ← L-DABA ← L-DABA ← **Z** ← L-Thr ← ← L-DABA ← R

	X	Y	Z
polymyxin A (M)	D-Leu	L-Thr	D-DABA
polymyxin B	D-Phe	L-Leu	L-DABA
polymyxin D	L-Leu	L-Thr	D-Ser
polymyxin E	D-Leu	L-Leu	L-DABA

Papain, ficin, Nagarse of *B. subtilis* and collistinase of *B. collistinus* catalyse the hydrolysis of peptide bonds in the side chain of the macrocyclic structure of poly-

Abbreviations: DABA, α,γ-diaminobutyric acid; DFP, diisofluorophosphate; PCMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetraacetate.

myxin B and polymyxin E (Paulus & Gray, 1964). The two latter enzymes were proved to be identical on immunochemical testing (Ito *et al.*, 1966). Degradation of polymyxin E (collistin) by the protease produced by the same organism results in a marked decrease in concentration of the desired fermentation product (Ito *et al.*, 1966). This prompted us to investigate properties of the proteases of *B. polymyxa* closely related to *B. collistinus*, and specificity of the isolated enzymes towards polymyxins. It is characteristic for the polymyxin-producing strains that biosynthesis of antibiotics of this group is not accompanied by formation of a family of related polymyxins differing in the amino acid constituents.

MATERIALS AND METHODS

Organisms and culture conditions. The mucoidless mutant d/13 isolated by Emilianowicz-Czerska *et al.* (1972) from *B. polymyxa* K-1 producing polymyxin E, and *B. polymyxa* ATCC 10401 were grown in 500 ml Erlenmayer flasks as described by these authors, or in the 14 litre Brunshwick fermentor as reported by Szcześniak, Brodowicz and Karabin (in preparation).

Isolation of proteases. The enzymes were isolated from the supernatant of the 30 h cultures by salting out with solid ammonium sulphate (730 g per litre). The precipitate was centrifuged for 15 min at 13 000 *g*, dissolved in water to contain 20 mg of protein/ml, acidified to pH 6.0 and a saturated solution of lead acetate was added dropwise to a final concentration of 0.06 M. The mixture was stirred for 30 min at 0°C. The resulting precipitate was centrifuged off for 10 min at 28 000 *g* and discarded. From the supernatant the enzyme was precipitated with ammonium sulphate at 0.4 saturation at pH 6.0 and purified on Sephadex G-100 using 0.05 M-tris buffer, pH 7.0, as an eluent.

Enzymic degradation of polymyxins. The isolated purified proteases were incubated at 40°C with polymyxin E, its degradation products, or polymyxin D alternatively in 0.4 M-tris buffer, pH 8.0 (final volume 1 ml) for estimation of residual antibiotic concentration, or in 0.05 M-borate buffer, pH 7.5, for chromatographic analysis of the degradation products.

Determination of proteolytic and esterolytic activities. Caseolytic activity was measured with 1 % casein in 0.4 M-tris buffer at pH 7.0. One unit was defined as that amount of the enzyme which resulted in $\Delta E_{280} = 0.001$ on a 40 min incubation at 40°C. Gelatin and albumin examined as the alternative substrates were used at the same concentration.

Elastolytic activity was measured after Ardelt (1968) using a suspension of fuchsin-stained elastin (5 mg/ml) in 0.4 M-tris buffer, pH 7.0. Incubation was carried out at 37°C for 18 h. The undissolved elastin was centrifuged off and extinction of the supernatant was read at 560 nm against control containing no enzyme.

Esterolytic activity was estimated using *N*-benzoyl-L-arginine ethyl ester after Schwert & Takenaka (1955).

Antibiotic concentration was measured by the microbiological cylinder-plate method with *Bordetella bronchiseptica* NCTC 8344 as a test organism.

Protein concentration was estimated after Lowry *et al.* (1951) with bovine serum albumin as a standard.

Hydrazinolysis of enzymic hydrolysate of polymyxin. A 60 min digest of polymyxin E (5 mg) dried under vacuum was suspended in anhydrous hydrazine (0.8 ml). Hydrazinolysis was carried out at 105°C for 6 h in the sealed tube according to Akabori *et al.* (1952). The excess of hydrazine was removed in the evacuated desiccator over sulphuric acid and the remaining mixture was shaken with benzaldehyde (0.5 ml). The separated aqueous phase was analysed by thin-layer chromatography.

Chromatography. Paper chromatograms were run on Whatman no. 3 paper in two alternative ascending systems: 1, butan-1-ol - pyridine - acetic acid - water (15 : 10 : 3 : 12, by vol.); 2, non-aqueous phase of butan-1-ol - butan-2-ol - 1 N-HCl mixture (3 : 15 : 20, by vol.). Thin-layer chromatography was carried out on plates covered with microcrystalline cellulose in the system: isopropanol - 85% formic acid - water (160 : 9.4 : 38.6, by vol.). Column chromatography of the enzymic hydrolysates of polymyxin E was performed on Whatman cellulose powder (column size: 1.5×40 cm) using non-aqueous phase of the following mixture: butan-1-ol - butan-2-ol - 2 M-acetic acid (8 : 4 : 20, by vol.). Separation was followed by measuring ninhydrin reaction after Alberti & Bartley (1963) at 575 nm.

Polyacrylamide disc electrophoresis. The 50-fold purified enzymic preparation from *B. polymyxa* (about 100 μg of protein) was subjected to electrophoresis in 0.5×7.0 cm tubes at 6 mA per tube according to Davis (1964). Gel rods were stained with Amido Black and the absorbance was recorded using Zeiss densitometer.

Reagents. Tris was purchased from Loba-Chemie (Wien, Fischamend, Austria), polymyxin E sulphate (16 000 units/mg) from Roger Bellon Laboratory (Neuilly, Seine, France) and polymyxin B sulphate (10 000 units/mg) from Pfizer (New York, U.S.A.). Polymyxin D (1200 units/mg) was obtained at the Institute of Antibiotics (Warsaw, Poland). Bacto-skim milk, dehydrated, was a product of Difco Laboratories (Detroit, Mich., U.S.A.) and microcrystalline cellulose of Applied Science Laboratories Inc. (State College, Pa, U.S.A.). Casein, polyacrylamide, TEMED, riboflavin, Amido Black were purchased from B.D.H. (Poole, Dorset, England) and cellulose powder for chromatographic use from Whatman Biochemicals Ltd (Maidstone, Kent, England). Protamine sulphate was from Koch-Light Laboratories Ltd (Colnbrook, Bucks., England), bovine blood serum albumin from Serva (Entwicklunglabor, Heidelberg, G.F.R.). Deacylated polymyxin E was a gift of prof. Kimura and fuchsin-stained elastin and elastase were from Dr. W. Ardelt (Institute of Rheumatology, Warsaw, Poland). Other reagents used were from Fabryka Odczynników Chemicznych (Gliwice, Poland).

RESULTS

Formation of polymyxin E and extracellular proteolytic activity in B. polymyxa *d/13 K-1*

Data given in Fig. 1 show that concentration of polymyxin E and the extracellular proteolytic activity increased rapidly up to the 18 h of growth. Later a steady but much slower increase of this activity was noted. At the same time an abrupt drop

in concentration of polymyxin E took place with simultaneous increase in pH to 7.0. Data on enzymic activity refer to the total proteolytic activity measured with casein as a substrate and may include several proteases of different physiological function.

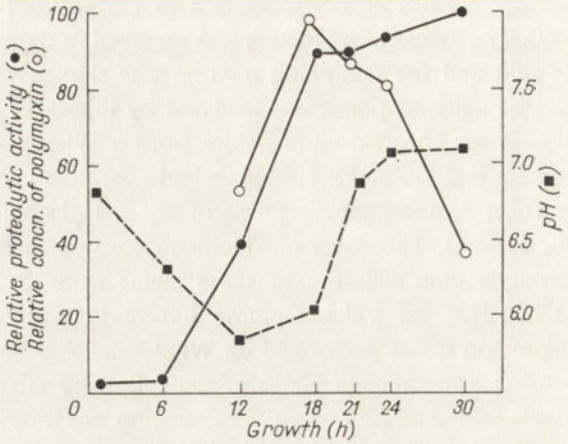

Fig. 1. Formation of polymyxin E (○) and extracellular proteolytic activity (●) during growth of *B. polymyxa* d/13 K-1. Samples were withdrawn from the 14 litre fermentor every 6 h during 30 h growth; proteolytic activity was determined with casein, and polymyxin concentration was estimated microbiologically as described under Methods. pH (■).

Characteristics of the isolated protease from B. polymyxa *d/13 K-1*

The protease was separated from the 30 h cultures of *B. polymyxa* and purified about 50-fold in three steps: by salting out with ammonium sulphate, treatment with lead acetate and chromatography on Sephadex G-100 (Table 1). The supernatant recovered after precipitation of the contaminating substances with lead acetate was concentrated by precipitating with ammonium sulphate before application to the Sephadex column.

Table 1

Purification of the extracellular protease of B. polymyxa *d/13 K-1*

Filtrate of the 30 h cultures was used. The enzymic activity was measured with casein at 40°C and expressed in units/40 min.

Purification steps	Volume (ml)	Activity		Purification factor	Recovery (%)
		specific	total $\times 10^{-3}$		
Culture filtrate	3 000	266	9 900		100
Ammonium sulphate sat. (precipitate)	350	790	8 400	3	85
0.06 M-Lead acetate treatment (supernatant)	335	1 300	6 950	5	70
Sephadex G-100	640	12 300	2 400	46	24

The isolated purified enzyme showed the following relative activity with the commonly used protein substrates: casein 100, gelatin 30, albumin 3, protamine 2 and elastin 0. On 2 h incubation of the purified enzyme (300 caseolytic units) at 40°C with polymyxin E (8000 units) in tris buffer, pH 7.0, inactivation of the antibiotic in about 90% took place (Fig. 2). Decomposition of antibiotic was linear up to 100 caseolytic units of the protease. In the range 300 - 2500 units the extent of inactivation was practically the same: the residual activity, about 10% of the initial, could be due to a slight antibiotic activity of one of the degradation products of polymyxin.

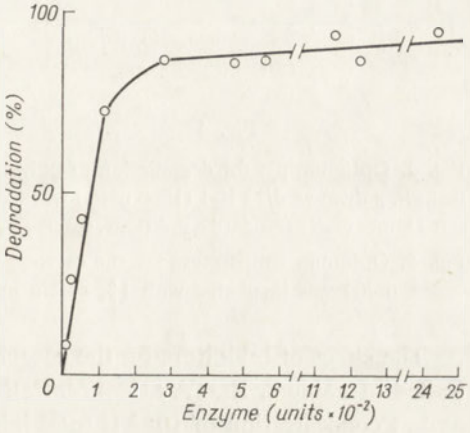

Fig. 2. Degradation of polymyxin E by the protease isolated from *B. polymyxa* d/13 K-1. The reaction mixture containing in the volume of 1 ml 0.2 M-tris buffer, pH 8.0, and polymyxin E (8000 units) was incubated with varying amounts of the enzyme for 2 h at 30°. The residual antibiotic concentration was estimated microbiologically.

Optimum pH for the caseolytic activity of the isolated protease was at 8.0 in tris-HCl or glycine-NaOH buffers (Fig. 3). At pH 7.0 this activity was only slightly lower while at pH above 8.0 a rapid decrease of the activity was observed. It is noteworthy that in phosphate buffer caseolytic activity was about 50% lower than in tris buffer, with distinct optimum at pH 7.0. The polymyxin-degrading activity of the protease in tris buffer, pH 8.0, was about 60% higher than at pH 7.0 (Fig. 4); above pH 8.0 polymyxin was unstable. Optimum temperature for the caseolytic activity on a 40 min incubation in tris buffer, pH 8.0, was 50°C (Fig. 5).

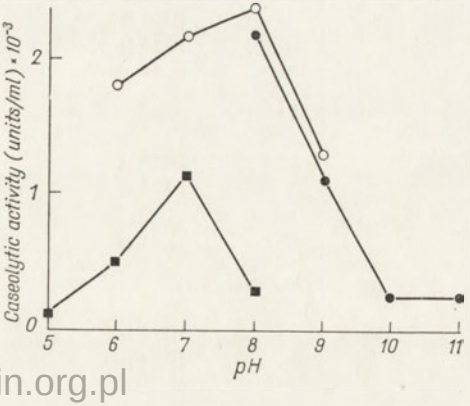

Fig. 3. Optimum pH of the caseolytic activity of the protease from *B. polymyxa* d/13 K-1. The purified protease (1500 units) was incubated with 1% casein for 40 min at 30°C in the following buffers: 0.1 M-tris-HCl buffer (○); 0.4 M-glycine--NaOH buffer (●); 0.4 M-phosphate buffer (■).

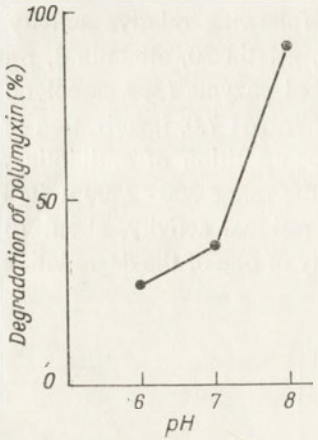

Fig. 4

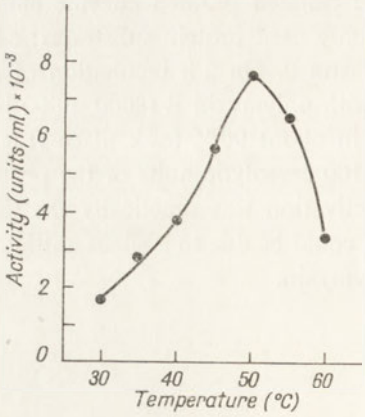

Fig. 5

Fig. 4. Optimum pH for degradation of polymyxin E by *B. polymyxa* protease. Protease isolated from *B. polymyxa* d/13 K-1 (1500 units) was incubated with polymyxin E (8000 units) in 0.4 M-tris -HCl buffer under standard conditions. Concentration of antibiotic was determined microbiologically.

Fig. 5. Optimum temperature for the caseolytic activity of *B. polymyxa* protease. Protease (1500 units) was incubated with 1% casein in 0.4 M-tris-HCl buffer, pH 7.0, for 40 min.

The effect of inhibitors on the caseolytic activity of the isolated protease is presented in Table 2. EDTA at concentration of 10^{-3} M inhibited this activity in 90% and at concentration of 10^{-2} M total inhibition was observed. Inorganic phosphate also showed an inhibitory effect, probably due to its metal chelating properties. DFP and PCMB were ineffective. The polymyxin-degrading activity of the protease was also inhibited by EDTA, i.e. the addition of EDTA to the incubation mixture

Table 2

Effect of inhibitors on the caseolytic activity of protease from B. polymyxa *d/13 K-1*

Incubation conditions see Methods.

Compound added	Inhibition (%)
EDTA	
10^{-4} M	69
10^{-3} M	89
10^{-2} M	100
P_i	
10^{-3} M	3
10^{-2} M	57
10^{-1} M	79
DFP	
10^{-3} M	0
10^{-2} M	20
PCMB	
10^{-5} M	0
10^{-4} M	0

protected polymyxin against protease action. In the presence of 10^{-2} M EDTA degradation of polymyxin E was reduced by 65% under standard assay conditions.

The addition of monovalent and divalent ions (Na^+, K^+, Ca^{2+}, Mg^{2+}) at 6.7×10^{-3} M concentration did not affect the activity and stability of the enzyme.

The investigated proteolytic preparation did not hydrolyse *N*-benzoyl-L-arginine ethyl ester.

Specificity of B. polymyxa *proteases*

The purified proteases isolated from the two strains of *B. polymyxa* showed high substrate specificity towards polymyxins. Polymyxin D was decomposed neither by protease of *B. polymyxa* d/13 K-1, synthesizing polymyxin E nor by the enzyme from *B. polymyxa* ATCC 10401 producing polymyxin D (Table 3) even when incubation was prolonged and was carried out overnight.

Table 3

Enzymic degradation of polymyxins

Polymyxin D or polymyxin E was incubated at 40°C for 40 min with the purified enzymic preparations (1000 caseolytic units). Concentration of antibiotic was determined using *Bordetella bronchiseptica*.

Source of protease	Substrate	Concentration of antibiotic (units/ml)		Decomposition (%)
		initial	after incubation	
B. polymyxa d/13 K-1 producing polymyxin E	Polymyxin E	7 920	550	94
	Polymyxin D	1 170	1 190	0
B. polymyxa ATCC 10401 producing polymyxin D	Polymyxin E	7 920	5 200	34
	Polymyxin D	1 170	1 100	0

The enzymes produced by either strain inactivated polymyxin E but with different effectiveness. In spite of the same caseolytic activity of both proteases in the incubation mixtures, the protease from the polymyxin E producing strain degraded polymyxin E in 90% upon 40 min incubation whereas the enzyme from the polymyxin D synthesizing strain inactivated polymyxin E in only 35%.

The time course of polymyxin degradation during 60 min incubation with proteases from *B. polymyxa* is illustrated in Fig. 6. The presented densitometric tracings of chromatograms of the degradation products sequentially released by the enzyme from the d/13 K-1 strain, indicate a two-step process. It seems also that the corresponding preparation from the ATCC 10401 strain catalysed only the first step of the antibiotic degradation.

The 60 min hydrolysate of polymyxin E gave on paper chromatography three spots (Fig. 6), whereas prolongation of chromatography to 24 h made possible

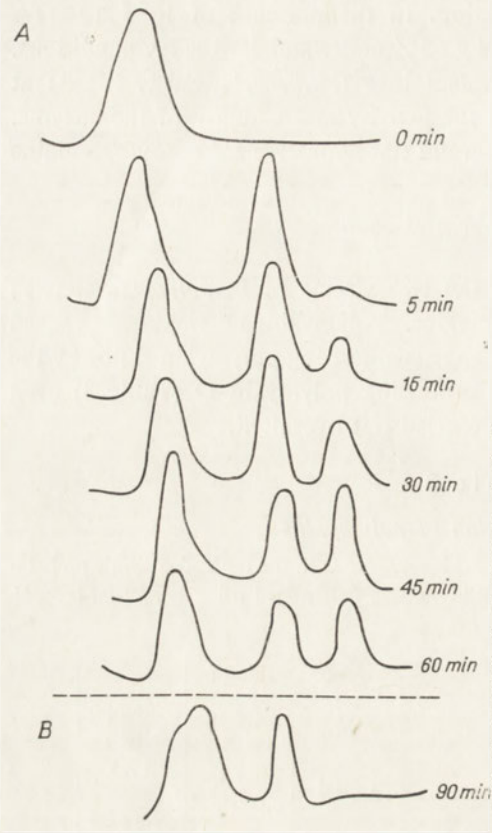

Fig. 6. Densitometric tracing of the degradation of polymyxin E by the protease from *B. polymyxa* d/13 K-1 (*A*) and protease from *B. polymyxa* ATCC 10401 (*B*). Polymyxin E (8000 units) was incubated with the respective protease (1500 units) in 0.05 M-borate buffer, pH 7.5. Samples were withdrawn at the indicated time intervals, deproteinized with perchloric acid, neutralized and run on Whatman no. 3 paper in the system: butan-1-ol - pyridine - acetic acid - water (15:10:3:12, by vol.). Chromatograms were developed with ninhydrin reagent containing 1 g of ninhydrin and 0.1 g of cadmium acetate in the acetone - acetic acid - water system (100:5:10, by vol).

Table 4

R_F *values of degradation products and amino acid constituents of polymyxin E*

Enzymic hydrolysate of polymyxin E obtained upon 60 min incubation with enzymic preparation from *B. polymyxa* d/13 K-1 was deproteinized with perchloric acid, neutralized and analysed by ascending chromatography on Whatman no. 3 paper in butan-1-ol - pyridine - acetic acid - water (15:10:3:12, by vol., system *A*) and in the upper phase of the butan-1-ol - butan-2-ol - 0.1 N-HCl mixture (3:15:20, by vol., system *B*).

Compound	R_F values in system	
	A	*B*
Polymyxin E	0.63	0.11
Polymyxin E, deacylated	0.26	—
Degradation products:		
I	0.66	0.26
II	0.47	0.02
III	0.42	—
IV	0.55	—
Leucine	0.55	0.45
Threonine	0.30	0.12
L-Diaminobutyric acid	0.19	0.02

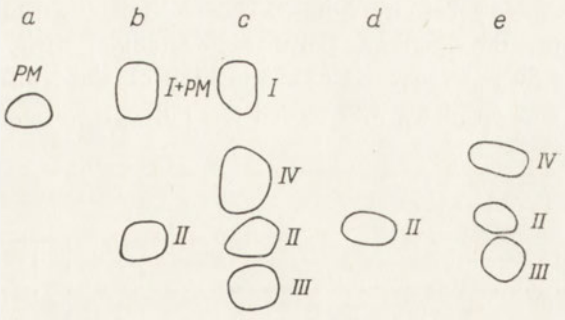

Fig. 7. Chromatography of the degradation products of polymyxin E (PM). Chromatograms of: polymyxin E (*a*); 5 min hydrolysate (*b*); and 60 min hydrolysate of polymyxin E (*c*); compound *II*, isolated from the 5 min hydrolysate of polymyxin E on column chromatography (Fig. 8), (*d*); enzymic hydrolysate of compound *II* (*e*). Chromatograms were run as described in the legend to Fig. 6.

detection of four compounds: *I*, *II*, *III* and *IV* (Fig. 7, Table 4). A compound of the same R_F value as compound *II* was detected in the 5 min enzymic hydrolysate. Separation of polymyxin from compound *I* in the 5 min hydrolysate was very poor in butan-1-ol - pyridine - acetic acid - water but could be easily performed when upper phase of butan-1-ol - butan-2-ol - 0.1 N-HCl mixture was used as the solvent system in paper chromatography (Table 4). By column chromatography on Whatman cellulose (Fig. 8) the 5 min hydrolysate of polymyxin E was resolved into three fractions corresponding to polymyxin E, compound *I* and compound *II*. The eluted fractions were checked and identified by paper chromatography. The isolated compound *I* was resistant to further enzymic hydrolysis while compound *II* on incubation with the enzymic preparation gave products corresponding to compounds *III* and *IV* found in the 60 min hydrolysate (Fig. 7). These results confirmed the two-step mechanism of polymyxin degradation. Degradation of polymyxin E did not result in liberation of DABA, threonine or fatty acid, since R_F values of these amino acids and that of deacylated polymyxin E differ considerably from R_F values of degradation products of polymyxin E. Neither was free fatty acid detected by gas chromatography in the enzymic hydrolysate (Dr. L. Lechowski, personal information).

DABA was identified on hydrazinolysis of the 60 min polymyxin hydrolysate as the *C*-terminal of the degradation products.

To see whether degradation of polymyxin is due to one or two enzymic proteins, the purified preparation from *B. polymyxa* d/13 K-1 was examined in polyacryl-

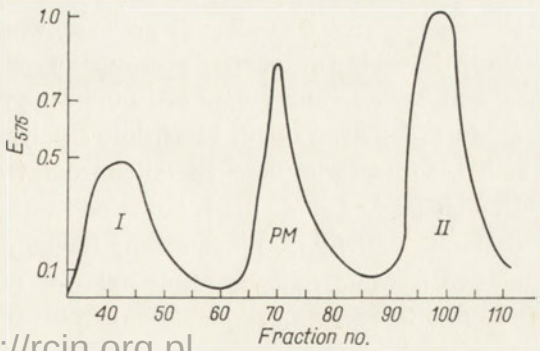

Fig. 8. Column chromatography of the 5 min enzymic hydrolysate of polymyxin E. The 5 min enzymic hydrolysate of polymyxin E (10 mg), obtained as described under Methods was applied on the cellulose column (1.5×40 cm) and eluted with the upper phase of the butan-1-ol - butan-2-ol - 2 M-acetic acid mixture (8:4:20, by vol.). Fractions of 1 ml were collected and ninhydrin reaction was measured at 570 nm. *PM*, polymyxin E. Compounds *I* and *II*.

amide gel electrophoresis. The densitometric reading of the stained rod was recorded and the casein and polymyxin degrading activity was localized, by slicing the parallelly run rods and examining the obtained discs on agar plates containing milk and on plates with seeded test microorganism. The results shown in Fig. 9 might

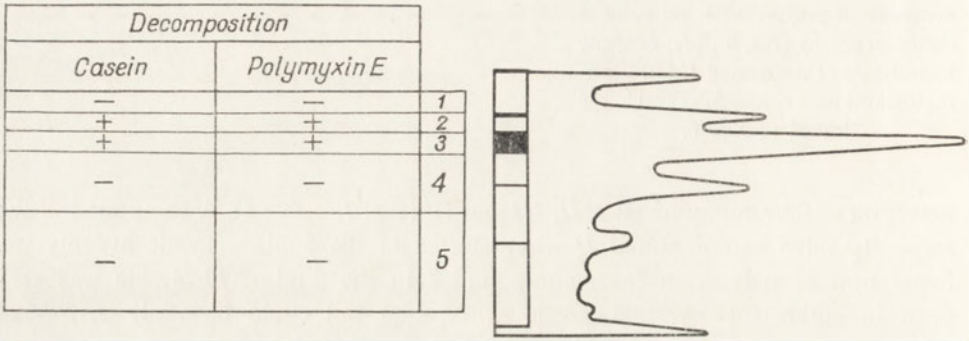

Fig. 9. Disc electrophoresis of the 50-fold purified proteolytic preparation from *B. polymyxa* d/13 K-1. Polyacrylamide gel electrophoresis was performed as described under Methods in triple tubes: one rod was stained with Amido Black, the other two were sliced as indicated. To localize the casein- and polymyxin-degrading activity the obtained discs were deposited on agar plates containing Bacto--skim milk and on the plates seeded with *Bordetella bronchiseptica*.

suggest the presence of two enzymes responsible for degradation of both casein and polymyxin. However, localization of proteolytic activity in correspondence to the particular protein bands is not very precise due to swelling of the gel rods on colour development.

DISCUSSION

The presented data on the properties of *B. polymyxa* protease indicate that it may be classified as a neutral metalloprotease (Cunningham, 1965). The enzyme is strongly inhibited by EDTA and shows much lower activity in phosphate buffer, in agreement with the observations of Griffin & Fogarty (1971). It should be also emphasized that EDTA at a concentration of 10^{-2} M protects polymyxin against decomposition by the protease. The sharp decrease of the proteolytic activity in the investigated preparation at pH above 8.0 and resistance to DFP excludes simultaneous occurrence of alkaline protease, which was observed in *B. subtilis* (Millet, 1970). Possibly two neutral proteases of very similar electrophoretic mobility are present in *B. polymyxa* d/13 K-1 producing polymyxin E, and both are active with casein and polymyxin E. Much lower polymyxin degrading activity per caseolytic unit was found with the preparation from strain ATCC 10401 producing polymyxin D. This might be due to: 1, "dilution" with the protease devoid of the polymyxin degrading activity, 2. lower affinity for polymyxin E, 3. presence of only one out of two enzymic proteins responsible for two-step degradation of the antibiotic. The results of chromatographic analysis of degradation of polymyxin E by the

enzymic preparation from *B. polymyxa* ATCC 10401 seem to support the third suggestion.

The proteases of *B. polymyxa* were found to be specific towards polymyxins; they decomposed polymyxin E but were inactive towards polymyxin D. Resistance of polymyxin D to proteolytic degradation *in vitro* is in agreement with the observed stability of this polymyxin in the cultures of *B. polymyxa* ATCC 10401 even upon prolonged cultivation (Emilianowicz-Czerska *et al.*, 1972). Nefelova *et al.* (1969) reported on the similar stability of polymyxin M, identical with polymyxin A.

The difference between polymyxin E and D consists in substitution of one leucine residue in the macrocyclic ring by threonine and replacement of L-DABA in the side chain by D-serine. Polymyxin E differs from polymyxin A in configuration of DABA in the side chain, and in replacement of leucine by threonine in the macrocyclic ring. This suggests the requirement of leucine and/or DABA for proteolytic action of *B. polymyxa* protease.

Degradation of polymyxin by plant enzymes: papain and ficin involves DABA residue in the side chain of polymyxin (Paulus & Gray, 1964). Suzuki *et al.* (1963) found that the site of polymyxin hydrolysed by Nagarse of *B. subtilis* is also the side chain but not the cyclic portion of antibiotic. Ito *et al.* (1966) on the basis of immunochemical studies considered protease of *B. collistinus* (collistinase) as identical with Nagarse and consequently believed that the mechanism of polymyxin degradation was the same as with plant enzymes and Nagarse. The lack of cross-reaction in immunochemical analysis could not be, however, an absolute criterion of the identity of the enzymes.

The results of our experiments clearly indicate a two-step degradation of polymyxin E by the preparation obtained from *B. polymyxa* d/13 K-1 producing this antibiotic. The mechanism involves splitting of polymyxin to compounds *I* and *II*, the latter undergoing further hydrolysis to compounds *III* and *IV*. It might be that two enzymes are responsible for this process, and possibly only one of them is present in *B. polymyxa* ATCC 10401 producing polymyxin D.

It should be also emphasized that DABA was found to be the *C*-terminal of the degradation products in the 60 min hydrolysate of polymyxin. This confirms lability to proteolysis of diaminobutyroyl residue in polymyxins.

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ROZKŁAD PROTEOLITYCZNY POLIMIKSYN PRZEZ ENZYMY *BACILLUS POLYMYXA*

Streszczenie

1. Z hodowli szczepu *B. polymyxa* d/13 K-1 wytwarzającego polimiksynę E (kolistynę) oraz z hodowli szczepu *B. polymyxa* ATCC 10401 syntetyzującego polimiksynę D wyodrębniono pozakomórkowe proteazy obojętne.

2. Enzymy oczyszczono około 50-krotnie stosując wysolenie siarczanem amonu, strącenie związków zanieczyszczających za pomocą octanu ołowiu oraz chromatografię na Sephadex G-100.

3. Oba enzymy rozkładają polimiksynę E lecz nie działają na polimiksynę D.

4. Podano ogólną charakterystykę enzymów i omówiono ich działanie na polimiksyny.

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CYCLOBUTANE DIMERS OF 1-METHYLTHYMINE: ISOLATION, IDENTIFICATION AND PROPERTIES

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All four stereoisomers of 1-methylthymine cyclobutane dimer were chromatographically isolated from photoproducts formed upon u.v. irradiation of the monomer in the solid state or in aqueous acetone. The stereochemical assignment of the different dimers was accomplished by comparison of the spectral (u.v., i.r.), chromatographic and some other physicochemical properties of their N_3 methylated derivatives with those of genuine samples of the known 1,3-dimethylthymine dimer isomers. A new photodimer of 1-methylthymine formed during acetone-sensitized reaction was isolated, partially characterized and tentatively identified as a photoreversible cyclobutanone--ring-containing adduct of ketene (from acetone photodecomposition).

Photodimerization of pyrimidines has been shown to be the main photochemical process leading to chemical and, in turn, conformational changes in DNA structure (Smith & Hanawalt, 1969; Varghese, 1972b). The appearance of these lesions in the genome of a living cell induces a series of dark repair processes, the inaccuracy of which may eventually result in some mutagenic changes (Smith & Hanawalt, 1969; Witkin, 1969).

In view of the biological importance of pyrimidine dimers their photochemical formation, structure and properties have been subject to a number of studies (Burr, 1968; Varghese, 1972b; McLaren & Shugar, 1964). In the last years we have been interested in the photochemical mechanisms of the diketopyrimidine dimer formation and in the effects of base stacking on the efficiency of photodimerization in polynucleotides (Tramer *et al.*, 1969), both in crystals (Lisewski & Wierzchowski, 1970) and in aqueous solutions of free bases (Lisewski & Wierzchowski, 1969, 1971; Stępień *et al.*, 1973; Stępień & Wierzchowski, in preparation). In this paper we give a more detailed account of some methods used for isolation and identification of 1-methylthymine photodimers in our study of the mechanism of their formation in water (Stępień & Wierzchowski, in preparation).

MATERIALS AND METHODS

Materials. 1-Methylthymine (1-MeT) was synthesized according to the Hilbert & Johnson (1930) method. It was purified by several cycles of crystallization from ethanol - ethyl ether (1 : 1, v/v) mixture to yield crystals melting at 282-3°C (uncorr.). Its purity was chromatographically confirmed in several solvent systems (Table 1).

Cis-syn and *cis-anti* 1,3-dimethylthymine dimers were obtained by irradiation of the frozen aqueous solution of 1,3-dimethylthymine and isolated according to Wulff & Fraenkel (1961). Owing to its insolubility in water, the *trans-anti* isomer precipitated during irradiation of a concentrated 0.1 M solution of 1,3-dimethylthymine (Morrison *et al.*, 1968). The precipitate was separated by filtration and after extensive washing with water appeared to be chromatographically pure. The fourth *trans-syn* isomer was prepared by methylation (according to Blackburn & Davies, 1966) of the *trans-syn* dimer of 1-MeT isolated as a single isomer from crystalline 1-MeT irradiated with a low dose of u.v. radiation (Lisewski & Wierzchowski, 1970).

Ion-echangers: Dowex 1-X8, formate form, 200-400 mesh and Dowex 50W-X2/H^+, 100-200 mesh, were purchased from Bio-Rad Laboratories (Richmond, Calif., U.S.A.) and spectrograde KBr from Merck (Darmstadt, G.F.R.). Reagent grade acetone was freshly distilled before use in photosensitized reaction. All solvents employed in chromatographic studies were products of Polskie Odczynniki Chemiczne (Gliwice, Poland). Water redistilled in glass was used.

Irradiation. To obtain larger yields of photodimers by means of the liquid phase irradiation, the acetone-sensitized triplet state dimerization was conducted in aqueous acetone (1 : 4, v/v) solution (1.7×10^{-2} M 1-MeT) as described in detail by Elad *et al.* (1967) and Lisewski & Wierzchowski (in preparation). Before irradiation and continuously during exposure, the solutions were flushed with oxygen-free nitrogen or argon. Irradiations were performed in a double-walled quartz vessel, water-thermostated at 25°C, using a Hanau Q700 mercury lamp and a glass filter (WG-7; 2mm thick, Schott-Jena) transmitting above 265 nm. Solutions were irradiated for about 28 h till about 85% of the monomer was converted into its respective photoproducts. High dimer yields were obtained also by irradiation of thin polycrystalline layers of 1-MeT: 0.04 M aqueous solutions of 1-MeT were slowly evaporated to dryness at room temperature on Petri dishes and then irradiated using the above-mentioned light source for up to 60 h.

Chromatography. a. Preparative separation of photoproducts. Irradiated aqueous acetone solutions of 1-MeT were evaporated on a rotary evaporator at about 30°C to remove acetone, and adjusted to pH 10.8 with concentrated (25 - 27%) aqueous ammonia. Samples containing 100 mg of dissolved solid photoproduct were, on account of its low solubility, at first adsorbed on 25 cm^3 of Dowex 1-X8 (formate form) which was then applied as top layer on a 2×45 cm column made of the same resin. Photoproducts were eluted in two steps with linear ammonium formate gradient (Weinblum & Johns, 1966). In the first step an oblique gradient G_1 was used: it was formed by placing in the mixing chamber 0.5 ml 85% HCOOH, 5 ml

conc. aqueous ammonia in 1 litre H_2O, and in the reservoir 1 ml 85% HCOOH, 7.5 ml conc. aq. ammonia in 1 litre H_2O. In the second step a steep gradient G_2 was applied: the mixing chamber contained 2.5 ml 85% HCOOH, 7.5 ml conc. aq. ammonia in 1 litre H_2O; reservoir 7.5 ml 85% HCOOH, 7.5 ml conc. aq. ammonia in 1 litre H_2O. 10 ml fractions were collected and the amount of u.v. absorbing material in the effluent was continuously recorded by means of the LKB Uvicord II absorptiometer. Small aliquots of the effluent were appropriately diluted with 0.1 N-NaOH and irradiated at 254 nm to monomerize dimeric photoproducts (Śmietanowska & Shugar, 1961; Sztumpf-Kulikowska *et al.*, 1967), till absorbance measured at 270 nm reached the maximum value. The amount of u.v. absorbing material at 270 nm in the effluent fraction was expressed in absorbance units. Usually not less than 80% of u.v. absorbing material was recovered from the column. Fractions corresponding to the individual peaks were pooled, checked for homogeneity using paper and thin-layer chromatography, desalted and purified as described by Weinblum & Johns (1966). The same procedure was applied to fractionation of photoproducts obtained from irradiated solid 1-MeT.

b. Analytical chromatography. Small scale separation of photoproducts for analytical purposes was carried out by ascending paper and thin-layer chromatography. For paper chromatography Whatman no. 1 paper was used. For the TLC technique silica-coated glass plates were made of kieselgel GF_{254} (Merck, Darmstadt, G.F.R.) or silufol (R) UV_{254} i.e. silica on aluminium foil (Kavalier, Czechoslovakia). Cellulose-coated plates were Eastman Chromatogram 6065 sheets. Five solvents used for development of chromatograms are given in Table 1.

Methylation of 1-methylthymine photoproducts. The purified 1-MeT dimers were methylated for further identification with dimethyl sulphate in 1 N aqueous NaOH, according to Wulff & Fraenkel (1961) and Blackburn & Davies (1966). In order to obtain higher chemical yields the reaction mixtures were cooled in ice. Methylation of photoproducts unstable in alkaline medium (dimer IV from solid state irradiation) was carried out either in 0.1 N-NaOH with dimethyl sulphate or in ethyl ether suspension using diazomethane. Methylation products were extracted with chloroform and purified by means of TLC preparative chromatography on kieselgel PF_{254} using chloroform-methanol (85 : 15, v/v) as a solvent. Spots corresponding to fully methylated products (the highest R_F values) were eluted with water, which was evaporated to dryness under vacuum, and the residual solid was dissolved in acetone. In order to remove all the contaminating fluorescein-indicator eluted with water from the TLC plates, acetone was evaporated and the compounds investigated were purified by vacuum sublimation. The methylated dimeric photoproducts were chromatographically homogeneous in all the chromatographic systems used. The following R_F values of 1,3-dimethylthymine dimers were obtained by TLC on silica gel GF_{254} using 12% ethanol in ethyl ether as a solvent: for *cis-syn*; *cis-anti*; *trans-syn*; *trans-anti* and monomer 0.19; 0.50; 0.62; 0.81 and 0.73, respectively (Lisewski & Wierzchowski, 1971). The melting points (uncorr.) of the respective dimers were: 254-9°C; 227-9°C; 257-9°C; 255-7°C and 148°C for the monomer. The present values are in

agreement with those given by Morrison *et al.* (1968). Melting points were measured by means of a Böetius microscope in the fused capillary tubes.

Determination of u.v. and i.r. spectra. All photoproducts as well as the methylation products were additionally purified by vacuum sublimation before determination of their u.v. and i.r. spectra.

The u.v. spectra of the aqueous solutions were measured in 10-mm silica rectangular cells with stoppers, using either a SP. 700 (Unicam) recording unit or a VSU-2 (Zeiss-Jena) manual spectrophotometer. The infrared spectra were recorded on a H.800 Hilger spectrophotometer in KBr pellets (about 0.5 mg per 200 mg of KBr).

RESULTS AND DISCUSSION

Isolation of 1-MeT dimers from the acetone-sensitized photoproduct. Typical column chromatographic elution pattern obtained for the acetone-sensitized 1-MeT photoproducts is shown in Fig. 1a. Elution with oblique gradient G_1 gave four major fractions referred to as X_1, I, II and III, which accounted for most of the photoproduct. After desalting and purification these fractions appeared chromato-

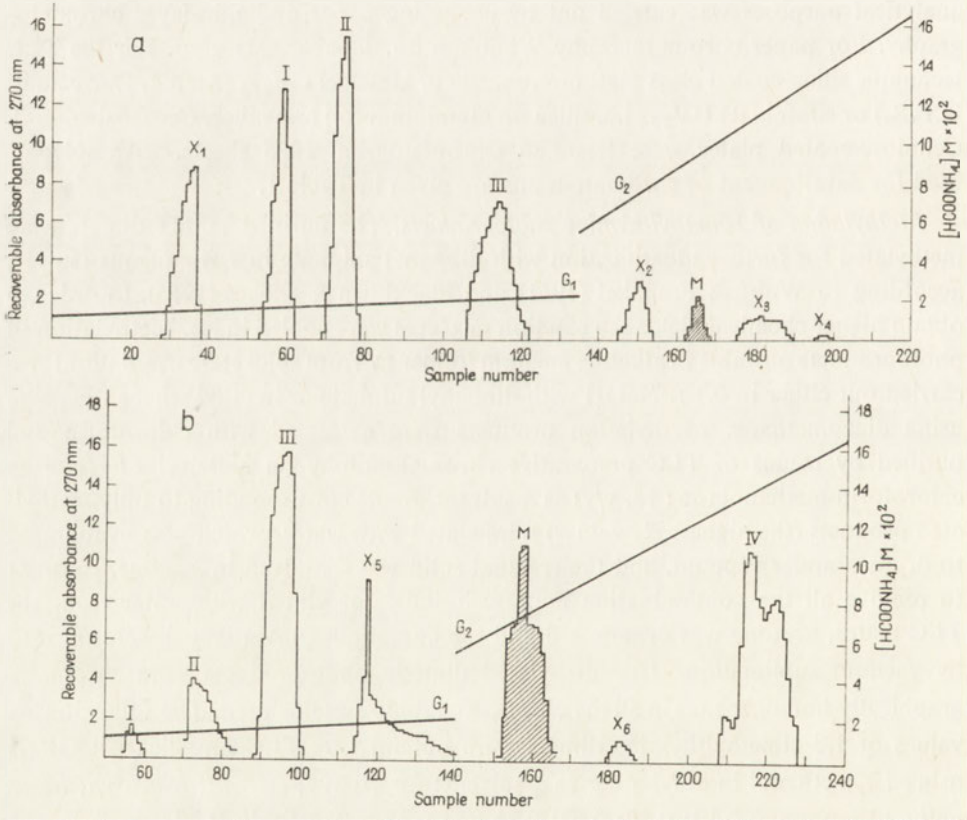


Fig. 1. Chromatographic separation of the 1-MeT photoproducts in G_1 and G_2 ammonium formate gradients: *a*, the acetone-sensitized reaction in aqueous acetone (1:4, v/v); *b*, irradiation of thin polycrystalline layers. (Details in the text).

graphically homogeneous and exhibited clearly defined R_F values in various paper and TLC chromatographic systems as shown in Table 1. The photoproducts obtained showed an absorption maximum in aqueous neutral solution at about 220 nm with the end absorption in the region of 270 nm (Figs. 2 and 5), i.e. at the absorption maximum of 1-MeT (Wierzchowski *et al.*, 1965). In alkaline medium (0.1 N-NaOH) the 220 nm absorption band underwent bathochromic shift to about 245 nm with

Table 1

R_F values and melting points of 1-methylthymine photoproducts

Solvents: 1, BuOH - CH_3COOH - H_2O (80:12:3, by vol); 2, isopropanol - NH_4OH - H_2O (7:1:2, by vol.); 3, *n*-propanol - H_2O (7:3, v/v); 4, isobutyric acid - H_2O - glac. acetic acid (100:50:1, by vol.); 5, chloroform - methanol (85:15, v/v).

Photoproduct	Whatman no. 1		Silica gel GF_{254}		Silufol UV_{254}		Cellulose plates (Eastman-Kodak)				M.p. (°C)
	1	2	1	5	1	5	1	2	3	4	
X_1	0.80	0.94	0.60	0.69		0.46	0.96	0.94	0.86		162
I (*cis-syn*)	0.72	0.35	0.35	0.36	0.35	0.36	0.47	0.64	0.63	0.69	decomp. 327 - 330
II (*cis-anti*)	0.83	0.51	0.48	0.53	0.45	0.56	0.62	0.80	0.75	0.76	decomp. 300
III (*trans-syn*)	0.79	0.48	0.50	0.56	0.47	0.60	0.56	0.76	0.70	0.83	decomp. 335 - 340
IV (*trans-anti*)	0.68	0.73	0.57	0.60	0.57	0.62	0.70	0.72	0.73	0.59	decomp. 295 - 300
X_2							0.62	0.68			
Monomer			0.55	0.58	0.55	0.58	0.74	0.77			282

a concomitant increase in the absorbance intensity (Figs. 2 and 5; Table 2). Irradiation of both neutral and alkaline solutions with the resonance 245 nm line of a low pressure Hg germicidal lamp brought about a fast increase in absorbance in the region of 270 nm, in a way typical for photoreversal of cyclobutane diketopyrimidine dimers to the parent monomers (Śmietanowska & Shugar, 1961; Sztumpf-Kulikowska *et al.*, 1967). The u.v. absorption spectra of fully photoreversed products

Table 2

The maxima of u.v. absorption spectra and molar extinction coefficients of 1-methylthymine dimers

Solvent	I (*cis-syn*)		II (*cis-anti*)		III (*trans-syn*)		IV (*trans-anti*)	
	λ(nm)	ε	λ(nm)	ε	λ(nm)	ε	λ(nm)	ε
H_2O	210	6690	210	6750	210	7570	210	9035
	215	5630	215	6320	215	6620	215	8530
	220	5280	220	6100	220	6150	220	8460
	225	5100	225	5880	225	5680	225	8040
0.1 N-NaOH	243	11 300	243	9470	243	12 900	245	8450

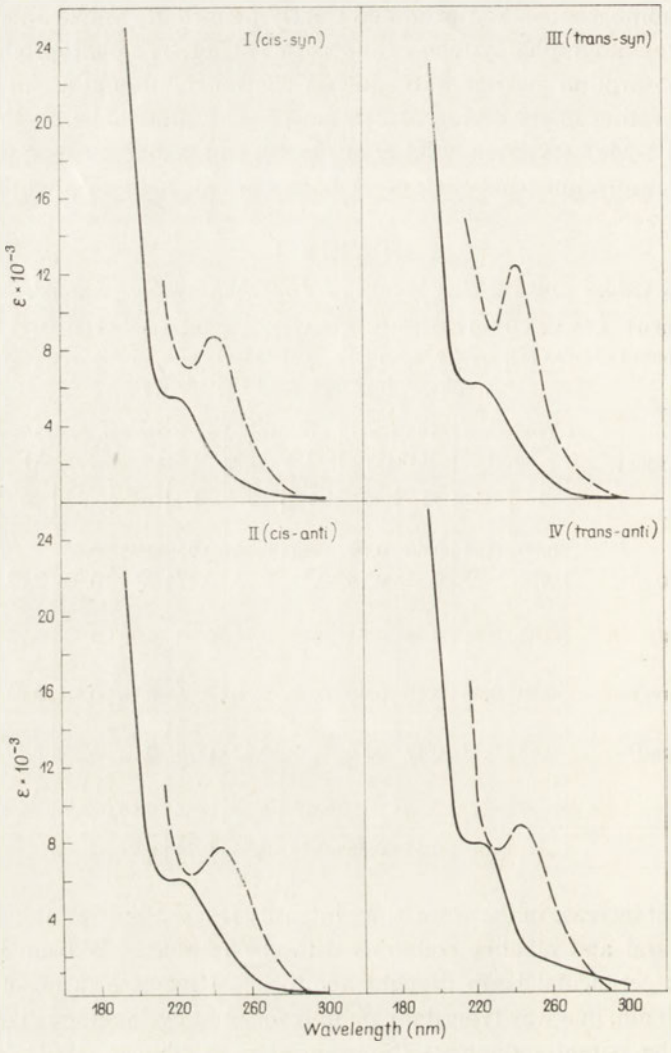


Fig. 2. Ultraviolet absorption spectra of 1-MeT dimers in aqueous neutral solution (——) and in 0.1 N-NaOH (- - -).

closely corresponded both at neutral and alkaline pH to those of 1-MeT (Wierzchowski *et al.*, 1965) (irrespective of the fraction number). Moreover, the chromatographic mobilities of each compound formed upon photoreversal appeared identical with those of 1-MeT, in the systems shown in Table 1.

On elution of the column with steep gradient G_2 three additional fractions of photoproducts were collected (X_2, X_3, X_4) apart from fraction M containing the unreacted 1-MeT. X_2 is a material with the absorption maximum in aqueous neutral solution at 265 nm; this material readily decomposed upon vacuum sublimation at 200 - 220°C with formation of 1-MeT. On u.v. irradiation at 254 nm or alkalization with 0.1 N-NaOH, the absorption spectrum of X_2 underwent irreversible batho-

chromic shift with formation of a broad asymmetrical absorption maximum at about 290 nm. The action of both agents appeared additive. X_3 and X_4 contained minute amounts of material exhibiting the absorption maximum at about 300 nm. Since we were in the first place interested in isolation of the cyclobutane dimeric photoproducts and the last three fractions contained negligible amounts of these photoproducts, no attempts to characterize further their chemical nature were undertaken.

Contrary to Jennings *et al.* (1970), who claim that the distribution of photoproducts of the acetone-sensitized reaction depends on the radiation dose, we observed no changes in the elution pattern of the progressively formed photoproducts. The same was found when samples were irradiated in a glass photochemical reactor transmitting light of wavelength >320 nm, using the same medium pressure mercury lamp with a WG-7 filter.

Isolation of dimers from irradiated solid 1-MeT. As it can easily be inferred from comparison of the elution profiles shown in Figs. 1a,b, the distribution of various photoproducts formed upon irradiation of crystalline 1-MeT significantly differs from that found in the acetone-sensitized photolysate. While elution with gradient G_1 gave fractions I, II, and III identical with those of the acetone-sensitized reaction, though in quite different relative amounts, the application of G_2 gradient resulted in isolation of the major fraction IV containing cyclobutane dimeric photoproduct. Its u.v. absorption spectra are shown in Fig. 2 and chromatographic mobilities — in Table 1. It is interesting to note that neither any fraction similar to X_1 nor any long wavelength absorbing photoproduct like X_3 and X_4 were found in the solid photolysate. Fractions X_5 and X_6 amounted to approximately 10% and 1%, respectively, of 1-MeT which was phototransformed in 80% under our experimental conditions. At lower u.v. doses X_5 and X_6 were hardly detectable. X_5 is the material exhibiting only the end absorption in the short wavelength of u.v. region and apparent photoreversion to 1-MeT upon irradiation at 254 nm. However, the i.r. spectrum of X_5 and its chromatographic properties significantly differed from those of the cyclobutane stereodimers of 1-MeT.

The number and relative amounts of various 1-MeT dimers formed in solid state strongly depended on the dose of absorbed u.v. light (Table 3). On up to 20%

Table 3

Distribution of the dimer isomers of u.v.-irradiated ($\lambda>265$ nm) polycrystalline 1-methylthymine at various degrees of monomer phototransformation

Phototransformed 1-MeT (%)	Stereodimers			
	I (*cis-syn*)	II (*cis-anti*)	III (*trans-syn*)	IV (*trans-anti*)
15 - 20	—	—	100	—
44	6	4	69	21
70	6	7	51	36
80	2	14	41	43

phototransformation of the monomer only one dimer corresponding to photodimer III is formed. As it can be predicted (Steward, 1963) from the molecular packing in monoclinic crystals of 1-MeT formed in water (Hoogsteen, 1963), this was the *trans-syn* isomer of 1-MeT dimer. On higher phototransformation of the monomer other isomers appeared. However, at the highest u.v. dose used, isomer IV was formed in an amount comparable to that of photodimer III. Since any mutual phototransformation of isomers in the solid state is highly unlikely, the observed dose-dependent distribution of photodimers can most satisfactorily be explained assuming that 1-MeT under conditions of thin-layer formation by slow evaporation of its aqueous solution crystallizes in two crystal modifications showing different molecular packings. In fact, it has been found by Hoogsteen (1963) that 1-MeT crystallizes from water to yield both stable monoclinic crystals of *trans-syn* dimer (Stewart, 1963), and its unstable modification showing so far unknown molecular packing. In view of the strong dependence of quantum yield of diketopyrimidine photodimerization on the distance between $C_5=C_6$ double bonds of neighbouring molecules in the crystal lattice (Lisewski & Wierzchowski, 1970), the two crystal modifications of 1-MeT may considerably differ in their ability to dimerize.

Identification of 1-MeT stereodimers. Assignment of stereochemical structure to different cyclobutane dimers of 1-MeT was accomplished indirectly in the following way. The purified samples of dimeric compounds from fractions I, II, III, IV and X_1 were treated with dimethyl sulphate under conditions permitting replacement of N_3 hydrogen atom by a methyl group to yield the corresponding dimers of 1,3-dimethylthymine. Methylation products were purified and characterized by determination of their chromatographic mobilities, i.r. spectra (Fig. 3a) and melting points (as described under Methods). Finally, their properties were compared with those of 1,3-dimethylthymine dimer standards of known conformation: *cis-syn* (Camerman & Camerman, 1968; Hollis & Wang, 1967), *cis-anti* (Camerman *et al.*, 1969; Hollis & Wang, 1967), and *trans-anti* (Morrison *et al.*, 1968). Samples of the three isomers of 1,3-dimethylthymine dimer were prepared and purified as described under Methods. It has been pointed out by Weinblum & Johns (1966) that dimers with the same cyclobutane linkages, e.g. 5-5, 6-6 (two *syn* isomers) or 5-6, 5-6 (two *anti* isomers) exhibit identical acid stability. Therefore, for better discrimination between *syn* and *anti* conformations of 1-MeT dimers, stabilities of fractions X_1, I, II, III and IV toward hydrolysis in concentrated perchloric acid and in 0.1 N-NaOH at 100°C (Table 4) were determined.

In this way it was unequivocally established that I, II and IV dimers correspond to *cis-syn*, *cis-anti* and *trans-anti* stereoisomers of 1-MeT dimer, respectively. The fourth possible configuration *trans-syn* was attributed to dimer III isolated from both the irradiated crystals of 1-MeT and the acetone-sensitized photolysate, taking into consideration the chemical and spectral properties as well as the topochemical criteria.

There was no doubt as to the presence of cyclobutane ring in III, in view of its u.v. absorption spectrum (Fig. 2) and of its ability to revert fully to 1-MeT on irradiation with short wavelength u.v. Like other 1-MeT cyclobutane dimers (I,

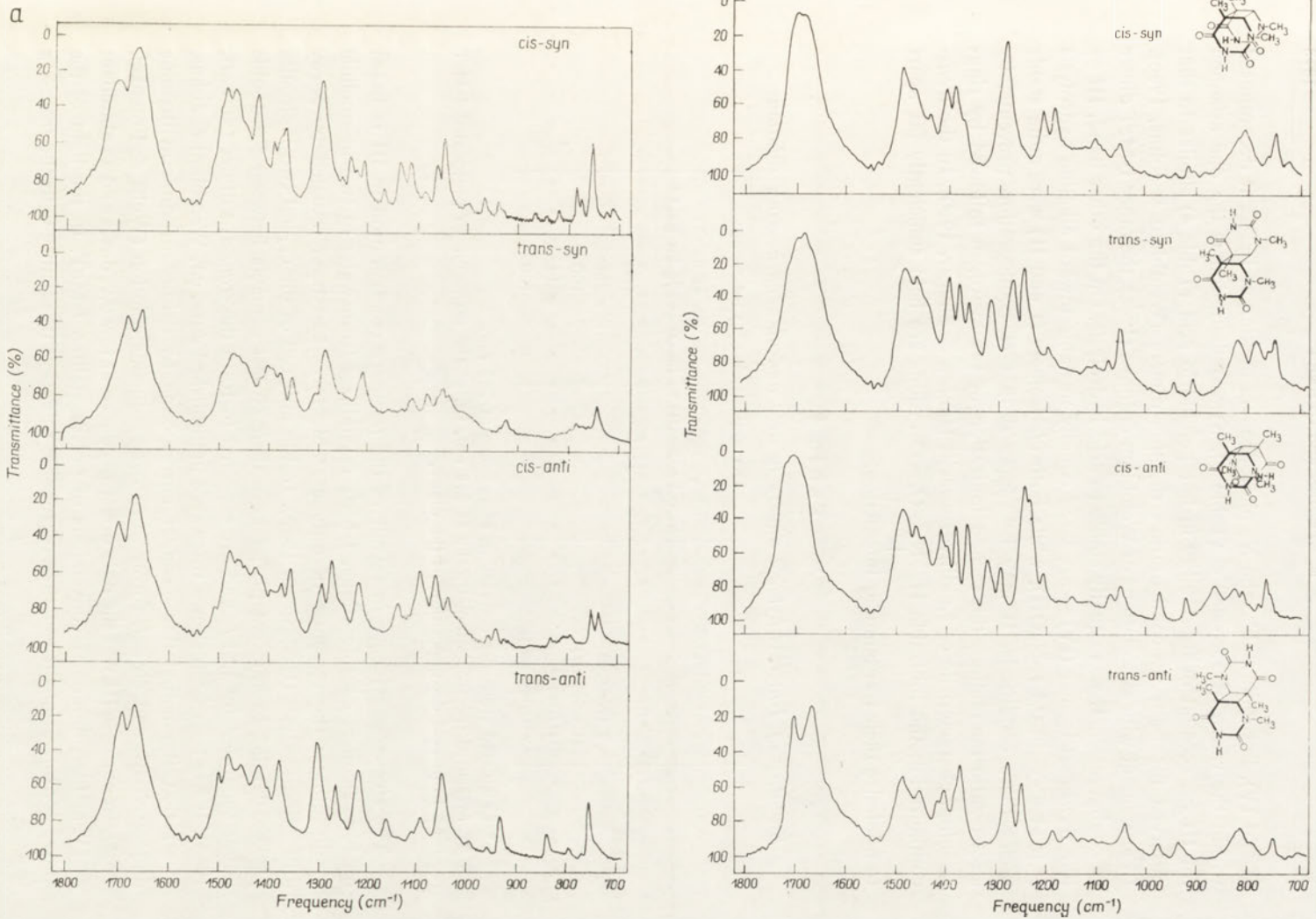


Fig. 3. Infrared spectra, in KBr pellets, of: *a*, stereoisomers of 1,3-dimethylthymine dimer; *b*, stereoisomers of 1-methylthymine dimer.

II and IV), but quite unlike the X_1 photoproduct melting at 162°C, it decomposed within the range of 335 - 340°C (Table 1). *Syn*-configuration is the one assumed for III on account of its stability in hot perchloric acid (Table 4) similar to that observed for both *syn*-configuration thymine dimers (Weinblum & Johns, 1966). In agreement with expectations of Weinblum & Johns (1966), the other 1-MeT dimer of configuration I, was equally stable to acid hydrolysis. On the other hand, III — like *trans-anti* isomer (IV) — appeared equally unstable toward alkaline hydrolysis (4 h at 85°C in 0.1 N-NaOH), while both *cis* isomers (I and II) were quite stable under these conditions. It may be supposed that *cis*-configuration prevents, by electrostatic interaction, the second ionization of N_3 hydrogen followed by ring-opening, characteristic of 5,6-dihydrouracils (Janion & Shugar, 1960). In the *trans*-configuration the ionizable $H—N_3—C_4=O$ groups are held sufficiently far apart to minimize their transannular interaction.

Table 4

Stability of 1-methylthymine dimers in acid and alkaline aqueous solutions

Photoproduct	Decomposition (%) following:	
	1 h at 100°C in 0.1 N-NaOH*	4 h at 85°C in 60 - 62% $HClO_4$**
X_1	44	100
I (*cis-syn*)	8	stable
II (*cis-anti*)	6	100
III (*trans-syn*)	50	stable
IV (*trans-anti*)	43	100

* Calculated from the decrease in absorbance at 245 nm.

** Measured spectrophotometrically at 278 nm, i.e. at the maximum of protonated 1-MeT resulting from acid decomposition of the dimer.

The strongest evidence in favour of the *trans-syn* configuration in III is based on topochemical considerations. 1-MeT crystallizes from water in two monoclinic modifications; the structure of only a stable form had been revealed by X-ray diffraction analysis (Hoogsteen, 1963). In the unit cell of the crystal two molecules packed parallel in adjacent planes have their closest contacts between 5,6 double bonds separated by 3.81 Å and are expected to form the *trans-syn* dimer (Stewart, 1963). It has later been shown (Stewart's unpublished data, c.f. Weinblum & Johns, 1966) that its methylation product corresponds to neither of dimethylthymine dimers *cis-syn* and *cis-anti* formed in frozen aqueous solution (Wulff & Fraenkel, 1961). In our case III was the only dimer produced at low u.v. doses and as abundant as IV at high monomer conversions of polycrystalline 1-MeT. In the light of the above considerations it was thus reasonable to expect its identity with the *trans-syn* isomer.

The i.r. spectra of 1-MeT dimers in KBr recorded for analytical purposes (Fig. 3b) exhibit a very distinct "fingerprint" pattern in the region of 1500 - 1200 cm^{-1}.

Characterization of the X_1 photodimer. We failed so far to identify with certainty the fifth dimer-like major photoproduct X_1 obtained in the acetone-sensitized reaction. X_1 comprised as much as 15% of phototransformed 1-MeT. In contrast to dimers I, II and III it was obtained from desalted evaporated effluent in the form of oil which was converted to crystals after several months of standing in the cold. The sublimed crystals melted at 162°C, i.e. at a temperature considerably lower than that of decomposition (above 300°C) of any one of the four 1-MeT dimers. Also the chromatographic properties of the purified product appeared quite different (Table 1); it exhibited the highest mobility of all dimeric species. It had, however, several properties in common with 1-MeT dimers. First of all, its u.v. absorption spectrum in aqueous neutral solution and in 0.1 N-NaOH, in spite of small bathochromic shift of about 10 nm (Fig. 4), closely resembles the spectra of I, II, III and

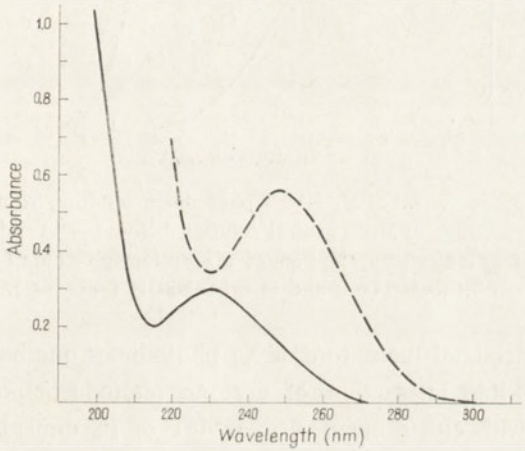


Fig. 4. Ultraviolet absorption spectra of X_1 photoproduct in aqueous neutral solution (——) and in 0.1 N-NaOH (- - -).

IV (Fig. 2) characteristic of the cyclic amide group in 5,6-dihydro-2,4-diketopyrimidines (Janion & Shugar, 1960; Wierzchowski *et al.*, 1965). On irradiation at 254 nm it apparently monomerized to 1-MeT both in alkaline, neutral and acid aqueous solutions (Fig. 5). In agreement with ionization data based on u.v. absorption, the i.r. spectrum of X_1 in chloroform solution contains a moderately strong band at about 3390 cm^{-1} characteristic of N - H stretch in diketopyrimidines (Kyogoku *et al.*, 1967).

The N_3 - H hydrogen atom could be replaced by a methyl group on alkylation with dimethyl sulphate in 0.1 N-NaOH. On irradiation at 254 nm methylated X_1 formed 1,3-dimethylthymine, as confirmed by the u.v. spectrum. Chemical analysis gave: C, 50.15%; H, 6.17%; N, 16.20%; a composition closely consistent with an adduct of 1-MeT dimer and acetone. However, any such covalently bonded structure can hardly be depicted. Formation of an unknown tetra-membered cyclic diether seems to be inconsistent with the stability of X_1 in dilute and concentrated acids.

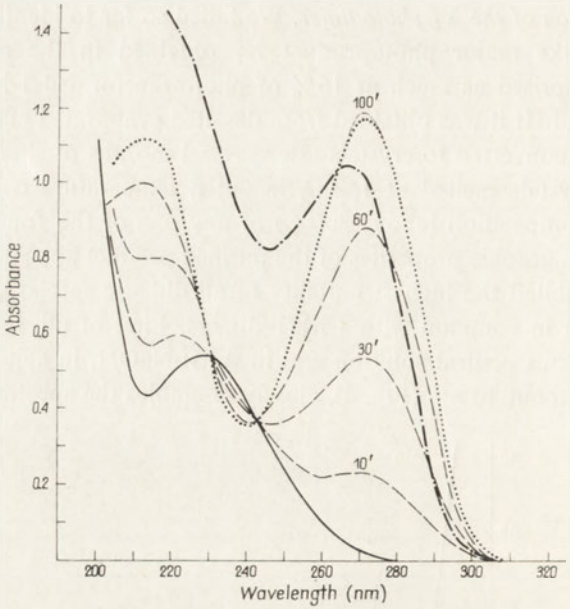
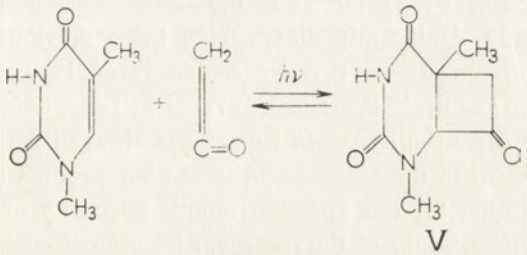


Fig. 5. The apparent photoreversal of X_1 1-MeT photodimer resulting from irradiation at 254 nm in water: absorption spectrum of X_1 in neutral solution before (——) and after exposure to the 254 nm radiation (- - -), as indicated in the Figure; absorption spectrum of fully photoreverted X_1 in 0.1 N-HCl (···) and in 0.1 N-NaOH (—·—·—).

In order to understand the nature of X_1 photodimer one has to take into consideration the possible interaction of acetone photodecomposition products in aqueous solution with either monomeric 1-MeT or its dimers. According to experiments of Volman & Swanson (1960), aqueous acetone is photochemically decomposed by a free radical mechanism involving production of ethyl, methyl and acetyl radicals. The disproportionation reaction involving the two last radicals eventually leads to the formation of methane and ketene, the latter being hydrolysed to acetic acid. The apparent photodimer X_1 may thus be a product of a radical or ketene attack on 5,6 double bond of 1-MeT. An attack of the transient acetone decomposition product on a dimer seems to be less probable on kinetic as well as chemical reasons. We were thus left with the hypothesis that X_1 is produced as a result of an attack of a transient acetone decomposition product on 1-MeT. Diketopyrimidine ring may serve either as a free radical scavenger like allyl alcohol (Volman & Swanson, 1960) or as an unsaturated system undergoing the cycloaddition reaction with ketene (Blomquist & Kwiatek, 1951). Since acetic acid, formed from ketene by hydrolysis (Volman & Swanson, 1960), is one of the major final products of acetone decomposition in aqueous solution, we would rather favour the second possibility. The more so that the diketopyrimidine 5,6 double bond is apt to undergo a variety of photocycloaddition reactions with unsaturated systems, including addition of a carbonyl group (Arnold, 1968; Barltrop & Carless, 1972), or addition of un-

saturated hydrocarbons like ethylene and propylene to uracil in aqueous solution (Pietrzykowska & Shugar, 1970; Krajewska & Shugar, 1972), as well as the acetone-sensitized cycloaddition of vinyl compounds and ketene diethyl-acetal to 1,3-dimethyluracil (Hyatt & Swenton, 1972). In the light of the latter study addition of a ketene molecule to the excited 1-MeT would most probably lead to the formation of cyclobutanone derivative V (Scheme 1), the structure of which seems not to be



V

Scheme 1

inconsistent with the spectral and chemical properties of X_1. It has recently been demonstrated (Carless *et al.*, 1972) that *cis-* and *trans-* 2,3 - and 2,4-dimethylcyclobutanones decompose photochemically to an olefin and a ketene. Cyclobutanone derivative V may thus be expected to behave similarly and to photodissociate with formation of 1-MeT and ketene. In fact photodecomposition of X_1 gives 1-MeT (Fig. 5). At the same time, an irradiated aqueous neutral solution of X_1 becomes acidic owing to formation of an organic acid, detected by gas chromatography. This last observation supports assignment of structure V to X_1, since formation in a primary photochemical step of ketene would in turn result in the appearance of acetic acid as a result of a fast hydrolysis of ketene in aqueous solution. Further experiments to examine the validity of this assignment are under way and will be reported separately.

Comments on photochemistry of 1-MeT. Previous reports on irradiation of 1-MeT in solid state contain somewhat conflicting evidence concerning stereochemistry of dimeric species. Blackburn & Davies (1966) have been able to show that of the two dimers formed in ratio 3 : 1 in frozen aqueous solution of 1-MeT the more abundant one showed *cis-syn* stereochemistry, while the other one was most probably the *trans-syn* isomer predicted by Stewart (1963). Their u.v. absorption spectra in 0.1 N-NaOH very well agree with those of dimers I (*cis-syn*) and III (*trans-syn*) identified in the present study. Goldstein (1967) has arrived at a similar conclusion studying photoproducts formed both in frozen aqueous 1-MeT solution and in crystalline 1-MeT; under the latter conditions the ratio between the two forms was reverted. On the other hand, Einstein *et al.* (1967) have isolated, from a frozen solution of 1-MeT, the least soluble dimer by precipitation in the cold and demonstrated its *trans-anti* configuration by determination of the X-ray crystal structure. In our experiments the *trans-anti* isomer appeared besides the *trans-syn* one in polycrystalline films of 1-MeT cast down by slow evaporation of an aqueous solution.

It may thus be inferred from all these data that 1-MeT is able to crystallize, in addition to the stable monoclinic form described by Hoogsteen (1963), in another one of quite different molecular packing giving rise to *cis-syn* and *trans-anti* photodimers. One of these two modifications corresponds to the unstable monoclinic form observed by Hoogsteen (1963). High yield of the *trans-anti* dimer observed in this study rather precludes its formation from pairs of randomly oriented molecules in non-crystalline regions of the solid film.

The distribution of 1-MeT dimers in the acetone-sensitized photolysate found in the present study significantly differs from those previously observed in the acetone-sensitized dimerization of thymine (Jennings *et al.*, 1970), thymidine (Ben-Hur *et al.*, 1967), uridine and uracil (Varghese, 1971) as well as cytidine (Varghese, 1972a). While in all these cases the *trans-anti* isomer has always been the major product, it was virtually absent in the acetone-sensitized photoproduct of 1-MeT. We have no explanation to offer for this apparent unique selectivity of 1-MeT photodimerization. Ready isomerization of the *trans-anti* isomer detected under the isolation conditions recommended by Khattak & Wang (1972) seems to rather be unlikely under mild conditions of thin-layer chromatography used in the present study.

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CYKLOBUTANOWE DIMERY 1-METYLOTYMINY: WYODRĘBNIENIE, IDENTYFIKACJA I WŁASNOŚCI

Streszczenie

Wszystkie cztery stereoizomery cyklobutanowego dimeru 1-metylotyminy wyodrębniono z fotoproduktu utworzonego po naświetleniu monomeru promieniowaniem nadfioletowym w fazie stałej i w roztworze wodno-acetonowym. Strukturę przestrzenną poszczególnych dimerów ustalono porównując widma absorpcyjne u.v. i i.r., własności chromatograficzne i temperatury topnienia N_3 metylopochodnych i wzorcowych stereoizomerów dimeru 1,3-dwumetylotyminy o znanej strukturze przestrzennej. Wyizolowano nowy fotodimer 1-metylotyminy utworzony w reakcji sensybilizowanej acetonem dimeryzacji, który częściowo scharakteryzowano i zaproponowano strukturę fotoodwracalnego adduktu ketenu (utworzonego w wyniku fotochemicznego rozkładu acetonu), zawierającego pierścień cyklobutanonowy.

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PHOTOCHEMISTRY OF 2,4-DIKETOPYRIMIDINES. PHOTODIMERIZATION, PHOTOHYDRATION AND STACKING ASSOCIATION OF 1,3-DIMETHYLURACIL IN AQUEOUS SOLUTION

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The initial quantum yields of 1,3-dimethyluracil photodimer (Φ_{df}) and photohydrate (Φ_{H_2O}) formation in aqueous solution were measured at various concentrations (1.6×10^{-3} - 1.1×10^{-1} M), in order to assess the influence of stacking association of the monomer on the efficiency of both reactions. Photohydration appeared concentration-independent, with $\Phi_{H_2O}=0.005\pm0.0003$ at 25°C. In the same concentration range photodimerization exhibited strong dependence on solute concentration and characteristic distribution of the stereodimers. Φ_{df} data were interpreted in terms of a model of singlet state dimerization by way of stacked complexes (Lisewski & Wierzchowski, 1971, *Mol. Photochem.*, **3**, 231 - 254), using an independently determined equilibrium constant of 1,3-dimethyluracil stacking association. This in turn permitted estimation of both quantum yield of dimer formation in complexes as being: $\Phi_{df}^{as}=0.028\pm0.004$, and establishment of the contribution of the triplet state photodimerization in the lower concentration range studied. Predominant formation of *trans-syn* and *cis-syn* dimers from stacked complexes was demonstrated as well. An about 2 - 3-fold difference in Φ_{df}^{as} between 1,3-dimethyluracil and 1,3-dimethylthymine is discussed in the light of the distribution of dimeric species in the photoproduct and of the efficiency of the radiationless process from the excited singlet state.

In our previous studies on the concentration, temperature and solvent effects on DMT[1] dimerization in aqueous solution we have shown (Lisewski & Wierzchowski 1969; 1971) that in the higher concentration range of the monomer ($>10^{-3}$ M) the reaction takes place predominantly by way of stacked association complexes. The involvement of a singlet excimer intermediate has been postulated in view of the high quantum yield of the reaction, which indicates a fast rate of dimerization step, comparable with the rate of radiationless processes. The same conclusion

[1] The following abbreviations are used in this text: DMT, 1,3-dimethylthymine; DMU, 1,3-dimethyluracil; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; DMCS, dimethylchlorosilane.

has independently been reached by Kleopfer & Morrison (1972) who investigated the initial quantum yield of dimethylthymine dimerization and the effect of triplet state quenchers thereon in various solvents. Earlier Fisher & Johns (1970) have reported also on the concentration and temperature dependence of thymine dimerization in aqueous solution.

In this light it seemed interesting to investigate the influence of stacking association on photochemical transformation of uracil derivatives undergoing in aqueous solution both dimerization and hydration reactions (Johns, 1969).

Moreover, we have taken into consideration our previous results (Wierzchowski & Shugar, 1959) which seemed to indicate a concentration dependence of quantum yield of photohydration of uracil derivatives. This was subsequently interpreted (McLaren & Shugar, 1964) as a result of formation of additional photohydrates during photodissociation of cyclobutane dimers.

MATERIAL AND METHODS

Chemicals. [2-^{14}C]1,3-dimethyluracil (DMU) was prepared by methylation of [2-^{14}C]uracil with dimethylsulphate according to Davidson & Baudish (1930). The product was purified by repeated crystallization from absolute ethanol - ether (1 : 1, v/v) and then additionally by vacuum sublimation (120°C; 1 mm Hg). The resulting [2-^{14}C]DMU showed final specific activity of 5.8 mCi/mmol. Freshly distilled acetone and benzene were used as solvents for TLC. Water redistilled in glass was used for preparation of DMU solutions.

Irradiation. A mercury-in-quartz medium pressure Q700 Hanau lamp cooled with distilled water circulating in a quartz jacket was used as a source of radiation. Combination of Schott-Jena glass filters WG-7 (2 mm) and UG-11 (2 mm), transmitting radiation in the range of 265 - 390 nm, was used for isolation of light absorbed within the long wavelength region of the absorption band of DMU. Quartz spectrophotometer cuvettes of 0.1 cm path length, with stoppers fitted with gas inlet and outlet tubes were used for irradiation. The cuvettes were placed in a brass thermostatting jacket at 26±1°C. The experiments were carried out in solution deaerated by bubbling a fine stream of argon for 30 min prior to irradiation (Jennings *et al.*, 1970). Argon was used also for mixing of the solution during irradiation. Traces of oxygen in argon were removed by three times repeated passage through an alkaline pyrogallol solution. In order to prevent changes in concentration of the DMU solutions argon was additionally passed through a gas washer containing water.

Chromatographic detection and determination of DMU photoproducts. Four DMU photodimer isomers and DMU photohydrate were separated and quantitatively determined by TLC and GLC. The TLC method was mainly used to determine the total content of dimers and photohydrate, while GLC served to separate the dimer isomers and to estimate their relative contents. Isolation of photoproducts by TLC chromatography was carried out on silica gel F_{254} plates (Merck, Darm-

stadt, G.F.R.). To achieve better separation, the DMU photoproducts were rechromatographed using benzene - acetone (7 : 3, v/v) as a solvent. Spots of the dimer isomers: *cis-syn*, *cis-anti*, *trans-syn* and *trans-anti*, of the photohydrate and DMU monomer (R_F values: 0.28, 0.30, 0.39, 0.56, 0.58, 0.60, respectively) were located autoradiographically on X-ray film (Foton, Warszawa) after about 150 - 180 h of exposure. Concentration of photoproducts were determined by radioactivity measurements using a Tricarb (Packard) liquid scintillation counter. Spots were cut out and quantitatively eluted with methanol, a good solvent for DMU photoproducts. Eluates were centrifuged at 15 000 r.p.m. and mixed with the scintillator solution (PPO 3 g+POPOP 0.1 g per litre of toluene) in the ratio 1 : 5 (v/v). It was found that at this methanol content quenching of the scintillator fluorescence did not exceed 30%. Under these conditions 25 - 30 μg of [2-^{14}C]DMU (specific activity 5.8 mCi/mmol) eluted from TLC spots were sufficient for quantitative determination in liquid scintillation counter. The radioactivity obtained was corrected for the mean background radioactivity.

The relative contents of stereodimers were determined using a Pye Unicam 104 gas chromatograph and a 1.5 m×0.5 cm metallic column packed with 10% Carbowax 20M on gas-chrom Z (acid-washed, DMCS-treated, 100 - 120 mesh). Isolation of dimers was carried out at 240°C with argon flow of 90 ml/min. Under these conditions the retention time for *trans-anti*, *trans-syn*, *cis-anti* and *cis-syn* DMU dimer stereoisomers was 45, 72, 84 and 137 min, respectively. The relative concentrations of dimers in the photolysate were measured as ratios of areas under the corresponding peaks. Molar concentrations of the different dimers were determined by comparison with a gas chromatogram of a standard solution of all four dimer isomers. The mean error of the GLC determinations amounted to about 15%.

Identification of DMU stereodimers. Stereoisomers of DMU dimer were indirectly identified by comparison of m.p., i.r. and NMR spectra, R_F values and stability toward acid hydrolysis (60% $HClO_4$, 100°C) of the isolated dimeric species with those previously reported for DMU dimers of known structure (Elad *et al.*, 1967; Fürst *et al.*, 1967; Rosenthal & Elad, 1968; Nnadi & Wang, 1969; Sasson *et al.*, 1970). DMU stereodimers were obtained on a preparative scale by sensitized dimerization of DMU in water-acetone solution (1 : 4, v/v) (Elad *et al.*, 1967) and subsequent isolation of the photoproducts by TLC. Attempts to separate the dimers according to Elad *et al.* (1967) were unsuccessful.

Determination of quantum yield. Measurements of the initial quantum yield of the hydration and dimerization reactions were based upon the radioactivity of [2-^{14}C]DMU photoproducts separated by means of TLC. Concentrations of various isomeric dimers were measured by gas chromatography as well.

The Φ_{dt} and Φ_{H_2O} values were measured for DMU conversions not exceeding 2%, i.e. under conditions in which phototransformation of 2,4-diketopyrimidine monomers is a linear function of the u.v. radiation dose (Johns, 1969; Whillans & Johns, 1969).

Quantum yield of photodimerization and photohydration was expressed in mol/einstein (M/E) and calculated from the formula:

$$\Phi = \frac{\Delta C}{\overline{\Delta L}}$$

where: ΔC is mol of photoproduct formed in the time interval Δt, and $\overline{\Delta L}$, the average fluence of light absorbed in the time interval Δt.

In vigorously stirred solutions every molecule is exposed to the same average fluence $\overline{\Delta L}$, which according to Johns (1969) is given by:

$$\overline{\Delta L} = I_0 \times \frac{f_{abs}}{2.3 \times A} = I_0 \times F \ (E \cdot min^{-1} \cdot cm^{-2})$$

Factor $f_{abs}/2.3\ \overline{A}$ (greatly dependent on the absorption of the solution) characterizes changes in $\overline{\Delta L}$ as a function of total absorption $\overline{A}$ of polychromatic light transmitted through filter and absorbed by DMU solutions. The incident light intensity I_0 was determined by uranyl oxalate actinometry, assuming a known average quantum yield of 0.54 for decomposition of oxalic acid (Leighton & Forbes, 1930). Concentration of the actinometer (aqueous solution of 0.03 M-$UO_2SO_4 \cdot 3H_2O$ + +0.15 M-$(COOH)_2 \cdot 2H_2O$) was chosen in such a way as to assure a practically complete absorption of the photochemically active radiation within a 1-cm layer of the solution.

The fraction of light absorbed by DMU solutions (f_{abs}) was calculated[2] on the basis of the relative distribution of the incident radiation energy emitted by the lamp, measured behind the filters:

$$f_{abs} = \frac{I_0^f - I_r^f}{I_0^f}$$

where: I_r^f is the intensity of radiation transmitted by the solution examined, and the incident radiation intensity is:

$$I_0^f = 4 \times 10^{-3} \times I_{265} + 2 \times 10^{-3} \times I_{270} + 1.9 \times 10^{-2} \times I_{280} + 1.5 \times 10^{-2} \times$$
$$\times I_{289} + 7.6 \times 10^{-2} \times I_{297} + 0.238 \times I_{302} + 0.646 \times I_{313}.$$

Values of I_r^f were calculated using the I_0^f data and known molar extinction coefficients (ε) of DMU at the wavelength of radiation transmitted through filters $\lambda(\varepsilon)$: 265 nm (8.8×10^3); 270 nm (8.5×10^3); 280 nm (4.7×10^3); 289 nm (7.4×10^2); 297 nm (3.8×10^2); 302 nm (6.05); 313 nm (1.0). The values of ε at the above mentioned wavelengths were calculated on the basis of the known value of $\varepsilon_{max} = 8.9 \times 10^3$ at $\lambda = 266$ nm, for aqueous solution of DMU (Shugar & Fox, 1952).

[2] We were unable to measure this fraction with satisfactory accuracy owing to the low absorption of light by a 0.1 cm layer of DMU solutions and low sensitivity of the uranyl oxalate actinometer.

The quantum yields for formation of the dimers and the hydrate[3], presented in Table 1, are mean values obtained from four independent irradiations at a given DMU concentration, each of which was followed by at least five TLC radiochromatographic determinations. Mean square errors of dimerization and hydration quantum yield did not exceed $\pm 11\%$ and $\pm 6\%$, respectively. The mean square error of the total quantum yield of the monomer conversion amounted to about 12%.

RESULTS

Initial quantum yields of photodimerization (Φ_{df}) *and photohydration* (Φ_{H_2O}) *of DMU in aqueous solution at various solute concentrations.* Quantum yield measurements were carried out in deaerated, thermostated (at 25°C) aqueous solutions, at several stoichiometric monomer concentrations ranging from 1.66×10^{-3} to 0.108 M. The measured values of quantum yields of total dimer formation (Φ_{df}) and of photohydration (Φ_{H_2O}) are given in Table 1 along with the calculated quantum yields of monomer disappearance $\Phi_{md}=2\,\Phi_{df}+\Phi_{H_2O}$. In agreement with our

Table 1

Initial quantum yield for dimers (Φ_{df}) *and hydrate* (Φ_{H_2O}) *formation and total monomer disappearance* (Φ_{md}) *of* 1,3-*dimethyluracil in aqueous solution*

Stoichiometric concn. of solute $[DMU]_0$	$\Phi_{df}\times10^3$	$\Phi_{H_2O}\times10^3$	$\Phi_{md}=(2\Phi_{df}+\Phi_{H_2O})\times\times10^3$
1.66×10^{-3}	0.43 ± 0.02	5.13 ± 0.28	6.00 ± 0.43
9.64×10^{-3}	0.64 ± 0.07	5.13 ± 0.22	6.42 ± 0.75
2.63×10^{-2}	1.08 ± 0.11	5.12 ± 0.11	7.28 ± 0.76
5.10×10^{-2}	1.75 ± 0.22	5.11 ± 0.11	8.60 ± 1.09
7.42×10^{-2}	2.63 ± 0.22	5.10 ± 0.18	9.83 ± 0.98
1.08×10^{-1}	3.26 ± 0.12	5.08 ± 0.32	11.62 ± 0.84

earlier observations (Wierzchowski & Shugar, 1959), Φ_{md} increases with the stoichiometric concentration of the solute. Inspection of Φ_{df} and Φ_{H_2O} demonstrates that the photodimerization reaction is solely responsible for the increase in Φ_{md}. The latter reaction exhibits strong dependence on DMU concentration, as it was previously observed for DMT photodimerization within the same solute concentration range (Lisewski & Wierzchowski, 1969; 1971). At the same time Φ_{H_2O} remains practically independent of the monomer content and attains a constant mean value of $\Phi_{H_2O}=0.005\pm0.0003$ (Table 1). The Φ_{md} values for DMU previously reported

[3] Lack of monochromatization should not affect the efficiency of both photohydration and photodimerization reactions, since the former has been shown to be wavelength-independent (Brown & Johns, 1968), as well as presumably photodimerization from the singlet state in aggregates (Fisher & Johns, 1970).

by Burr *et al.* (1968; 1972) and Wang (1962) are in fair agreement with ours, if the differences in DMU concentration during determination of quantum yield are taken into account. However, the values of $\Phi_{md} \sim 0.01 - 0.014$ obtained by Moore & Thomson (1956), and more recently by Johns (1971) are about twice as high as those presently reported.

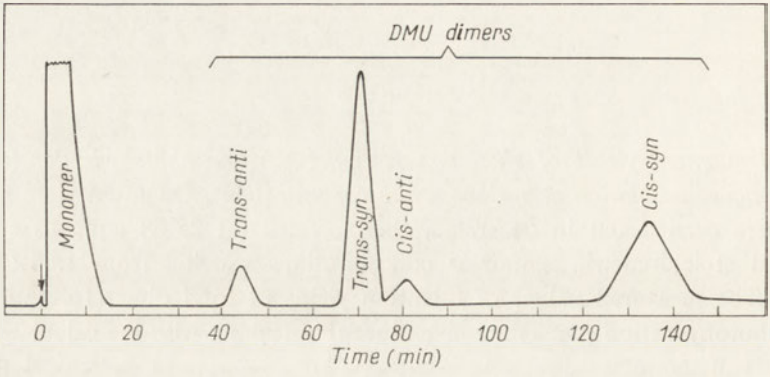

Fig. 1. Separation of 1,3-dimethyluracil stereodimers by gas chromatography. (Experimental conditions given in the text).

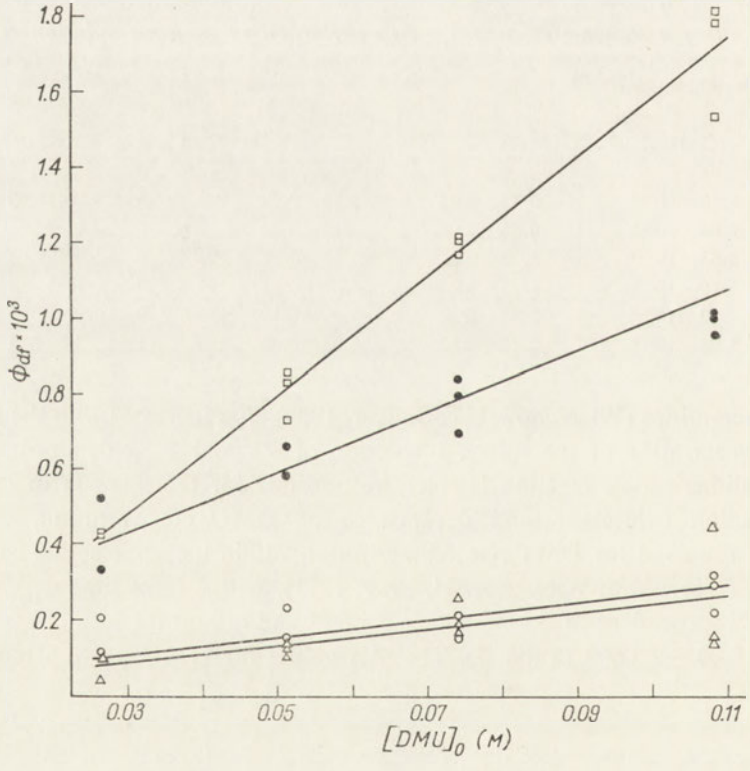

Fig. 2. Initial quantum yields of formation of various 1,3-dimethyluracil stereodimers as a function of the stoichiometric solute concentration; □, *trans-syn*; ●, *cis-syn*; ○, *trans-anti*; △, *cis-anti*.

Distribution of DMU stereodimers. The differences in stereoselectivity of the photo-cycloaddition of DMT from the singlet and triplet excited states (Kleopfer & Morrison, 1972; Morrison & Kleopfer, 1968), and the possible influence of the monomer stacking in ground state complex governing photodimerization at higher concentrations in water (Lisewski & Wierzchowski, 1969; 1971; Fisher & Johns, 1970), prompted us to study the distribution of the four possible DMU stereodimers (Wulff & Fraenkel, 1961).

Typical gas-chromatographic separation of DMU dimers formed in 0.108 M-DMU solution after 2.2% photoconversion of the monomer is shown in Fig. 1. Formation of four DMU stereodimers was observed over whole DMU concentration range studied. Their distribution, however, showed characteristic variations with stoichiometric concentration of the monomer. These data are presented in Fig. 2 as plots of quantum yields of individual dimer formation *versus* stoichiometric DMU concentration. It is clearly seen that formation of the two isomers *trans-syn* and *cis-syn* exhibits strong concentration dependence, while the *trans-anti* and *cis-anti* isomers are produced with lower efficiency almost independently of the actual concentration of DMU solution. Consequently, in the highest concentration range the two former isomers account for as much as 90% of the total dimer photoproduct. Quite different distribution of dimeric species has been observed in the case of photodimerization in aqueous solution of DMT (Lisewski & Wierzchowski, 1969; 1971; Kleopfer & Morrison, 1972) and thymine (Fisher & Johns, 1970).

DISCUSSION

Effects of DMU concentration on photodimerization and ground state stacking association of the monomer. It is at present well established that photodimerization of diketopyrimidines in aqueous solution at low ($\leqslant 10^{-4}$ M) solute concentration involves a bimolecular diffusion-controlled reaction between a molecule in the excited triplet state and the one in the ground state (Sztumpf-Kulikowska *et al.*, 1967; Fisher & Johns, 1970). It can easily be shown (see for instance Lisewski & Wierzchowski, 1971) that at concentrations at which radiationless inactivation (k^3_{nr}) of the triplet state molecules becomes negligible, as compared with the sum of their photochemical (k^3_{df}) and self-collisional quenching (k^3_{sq}), the quantum yield of this reaction Φ^T_{df} attains a maximal concentration-independent value $\Phi^T_{df}(\max) = \Phi_{isc} \cdot k^3_{df}/(k^3_{df} + k^3_{sq})$. At higher monomer concentrations a second, much more efficient photodimerization mechanism is simultaneously involved by way of excitation of ground state stacked association complexes, as it has been demonstrated in a previous study on photodimerization of DMT (Lisewski & Wierzchowski, 1969; 1971). This has later been confirmed by Kleopfer & Morrison (1972) for the same system as well as by Fisher & Johns (1970) for thymine. The efficiency of this mechanism of photodimerization is entirely governed by stacking association equilibrium and thus exhibits strong dependence on the concentration of associated monomers. Assuming the multi-step model of stacking association

in water (Ts'o *et al.*, 1963), the measured quantum yield of dimer formation can be shown (Lisewski & Wierzchowski, 1971) to be the product of quantum yield of dimer formation within stacked complexes Φ_{df}^{as} and the fraction of associated solute molecules:

$$\Phi_{df} = \Phi_{df}^{as} (2 K_{as} B_1 - K_{as}^2 B_1^2) \quad (1)$$

where: K_{as} is the stacking association constant assumed to be equal for all stages of multi-step association; B_1 is the concentration of non-associated monomers, and the expression in the bracket gives the $(B_0 - B_1)/B_0$ fraction of associated monomers; B_0, the stoichiometric concentration of solute.

Association equilibrium constants for stacking interaction of DMU $K_{as}^{25°} = 0.70 \pm 0.04$ litre · mol^{-1} and of other diketopyrimidines in aqueous solution have recently been determined by equilibrium ultracentrifugation and thermal osmometry (Plesiewicz & Wierzchowski, 1972). The experimental Φ_{df}'s from Table 1 can be plotted using this K_{as} value according to Eq. 1, as shown in Fig. 3. Quantum

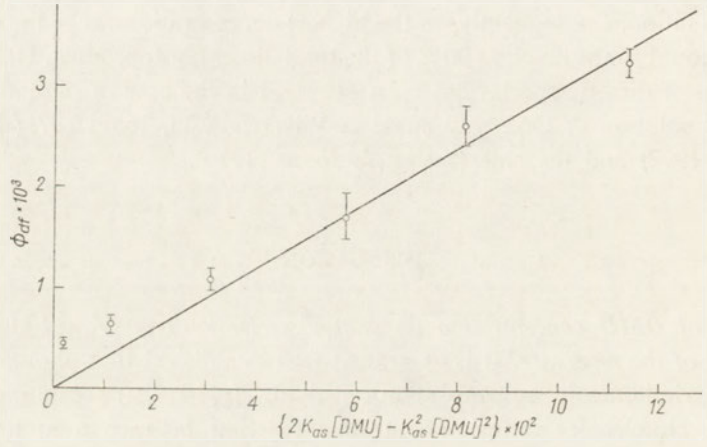

Fig. 3. Plot of initial quantum yield of the total of 1,3-dimethyluracil dimer formation *versus* fraction of the associated solute molecules according to Eq. 1.

yields corresponding to low concentrations do not seem, however, to fit very well the calculated linear plot which according to Eq. 1 should pass through the origin of coordination axes. Their upward deviation from that line strongly suggests simultaneous involvement of the triplet state reaction. In fact, measurements of Φ_{df} at the lowest DMU concentration studied (1.6×10^{-3} M) in oxygen-saturated solutions qualitatively demonstrated involvement of an oxygen-quenchable reaction $\Phi_{df}/\Phi_{df}^{O_2} \leqslant 1.6$. This has not been observed in the case of photodimerization of DMT, the triplet state dimerization of which reaches the maximum efficiency $\Phi_{df}^{T}(\max) = 4 \times 10^{-5}$ in an about 10^{-4} M solution contributing but little to the total photodimer formation at DMT concentrations exceeding 10^{-3} M (Lisewski & Wierzchowski, 1971). In this concentration range the quenching effect of oxygen upon Φ_{df}^{DMT} could hardly be detected.

Provided that concentration of stacked species in DMU solutions are governed by this same multi-step model of association, the ratio of experimental quantum yields $R = \Phi_{df}^{DMT}/\Phi_{df}^{DMU}$ should, according to Eq. 1, be a constant value, if only photodimerization by way of stacked complexes would significantly contribute to photoproduct formation. On the other hand, R might be expected to be a sensitive index of the solute concentration under conditions of significant involvement of the triplet state reaction in DMU photodimerization. Using Φ_{df}^{DMT} values reported in our earlier paper (Lisewski & Wierzchowski, 1971) and Φ_{df}^{DMU} taken from Table 1, R was calculated and plotted as a function of the concentration of solute, as shown in Fig. 4. In the lower concentration range it exhibits a strong concentration de-

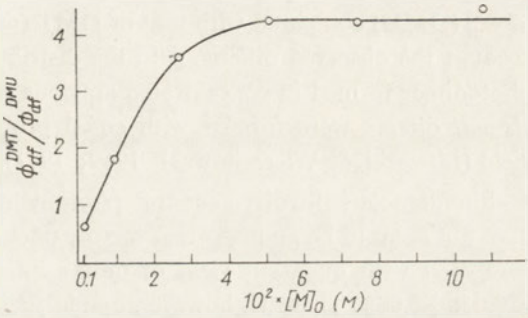

Fig. 4. Dependence of the $\Phi_{df}^{DMT}/\Phi_{df}^{DMU}$ ratio on the stoichiometric solute concentration in deaerated aqueous solution. Φ_{df}^{DMT} data taken from the previous paper (Lisewski & Wierzchowski, 1971).

pendence and leaves off at concentrations exceeding 4×10^{-2} M. Important contribution of the triplet state reaction in DMU photodimerization below this threshold concentration in thus clearly visualized. The high degree of this contribution in the case of DMU seems to be primarily due to the fact that the efficiency of dimer formation from stacked complexes is more than four times lower than that observed for DMT. The limiting quantum yield of the triplet state reaction Φ_{df}^{T} (max), being higher for DMU than for DMT, can be a factor as well, since the intersystem crossing efficiency in acetonitrile solution has been reported (Lamola & Mittal, 1966) to be four-fold greater in DMU than in DMT. On the grounds of the difference between $\Phi_{df} = \Phi_{df}^{T} + \Phi_{df}^{as}$ (measured at the lowest DMU concentration; Fig. 3) and Φ_{df}^{as} obtained by linear extrapolation of Φ_{df} according to Eq. 1, Φ_{df}^{T} (max) was assessed to be roughly 4×10^{-4}, i.e. by one order of magnitude greater than the corresponding value for DMT (Lisewski & Wierzchowski, 1971). From the slope of the plot in Fig. 3, the quantum yield of DMU dimer formation within stacked complexes $\Phi_{df}^{as} = 0.028 \pm 0.004$ can directly be obtained. In view of the distribution of various dimeric species shown in Fig. 2, this quantum yield clearly represents the mean value of quantum yields of the formation of individual dimers. Since the rate of dimerization within complexes $k_{df} \geqslant 10^{12}$ is much faster than the diffusion controlled rate of their dissociation, the distribution of photodimers may be expected to reflect the proportion of the relative populations of DMU molecules of various mutual orientations in their ground state stacked complexes. Thus, the slopes of Φ_{df} *vs* $[DMU]_0$ plots in Fig. 2 for the individual dimers correspond to different Φ_{df}^{as} for dimer formation from variously oriented pairs of monomer molecules. Only

the slopes of plots for both *syn*-fused dimers are sufficiently steep to suggest their exclusive formation from stacked complexes, the more so that these dimers account for more than 90% of the photoproduct at the highest DMU concentrations studied. The two *anti*-fused dimers are formed with a similarly low quantum yield exhibiting only slight concentration dependence. In view of the demonstrated important contribution of the triplet state reaction, these dimers seem to be produced largely by this mechanism. This does not mean, of course, that the other two dimers are not being formed with similar Φ_{df} from the triplet state. Observation of photoproduct distribution in the presence of triplet quenchers may be helpful in elucidating this question (Kleopfer & Morrison, 1972).

When considering the possible reasons underlying the 2-3 times lower value of Φ_{df}^{as} (DMU) compared with that of DMT ($\Phi_{df}^{as}=0.065\pm0.02$)[4] one has to take into account the observed difference in the distribution of isomers in DMU and DMT photodimer formed in concentrated aqueous solution. In the latter case *cis-syn* and *cis-anti* dimers were formed with equal probability and in about 80% chemical yield (Lisewski & Wierzchowski, 1969; 1971).

Simultaneous involvement of photohydration in photochemistry of DMU does not seem to be an important factor, since $\Phi_{H_2O}=0.005$ can be considered small compared with quantum yield of monomer disappearance in a complex $2\,\Phi_{df}^{as}=0.056\pm0.008$. There is, however, ample spectroscopic evidence that radiationless deactivation of singlet excited states of uracil and its derivatives is by far more efficient than in the case of thymines (Eisinger & Lamola, 1971). One would then expect that the relatively short life time of singlet excited state of DMU might primarily be responsible for the inefficiency of dimerization by way of a singlet excimer formed upon excitation of a ground state stacked complex (Lisewski & Wierzchowski, 1971).

Comments on photohydration of DMU. The independence of Φ_{H_2O} of DMU concentration in the whole concentration range studied can be taken as evidence that the probability of photoaddition of a water molecule to DMU does not *practically* depend on whether the reaction is taking place with a free or associated monomer molecule. In view of the low concentration of the associated species, not exceeding 15%, even at the highest DMU concentration, and bearing in mind the relatively large error ($\pm5\%$) involved in Φ_{H_2O} determination, we can not state with certainty that the stacking does not affect at all the rate of photohydration. These conclusions seem to be supported by the fact that the secondary structure of the poly(A)· poly(U) helical complex has no effect on quantum yield of photohydration of uracil residues in comparison with free uridylic acid in random-coil form (Johns *et al.*, 1966; Pearson & Johns, 1966). The present results indicate that

[4] Previously reported $\Phi_{df}^{as}=0.18$ (Lisewski & Wierzchowski, 1971) was estimated from high concentration equilibrium ultracentrifugation data using $K_{as}^{25°}=0.4\pm0.25$ litre·mol^{-1}. Recent thermal osmometric data (Plesiewicz & Wierzchowski, to be published) show that in view of strong anticooperativity involved in the first association step of DMT in the low concentration range ($\leqslant$0.1M) the association constant is better approximated by $K_{as}^{25°}=1.6\pm0.4$ litre·mol^{-1}.

our earlier observations (Wierzchowski & Shugar, 1959) on the effect of concentration on quantum yield of DMU and uridine disappearance, could not be interpreted (McLaren & Shugar, 1964) as being due to the formation of additional hydrates from their dimers during photodissociation.

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FOTOCHEMIA 2,4-DWUKETOPIRYMIDYN. FOTODIMERYZACJA, FOTOHYDRATACJA I WARSTWOWA ASOCJACJA 1,3-DWUMETYLOURACYLU W WODNYM ROZTWORZE

Streszczenie

Zmierzono początkową wydajność kwantową tworzenia fotodimeru (Φ_{df}) i fotohydratu (Φ_{H_2O}) 1,3-dwumetylouracylu (DMU) w roztworze wodnym przy różnych stężeniach DMU ($1{,}6\times10^{-3}$ - $1{,}1\times10^{-1}$ M) w celu zbadania wpływu warstwowej asocjacji monomeru na efektywność obu reakcji. Wydajność kwantowa reakcji fotohydratacji nie zależy od stężenia DMU w roztworze, przyjmując w badanym zakresie stężeń w temp. 25°C stałą wartość $\Phi_{H_2O}=0.005\pm0.0003$. Tymczasem reakcja fotodimeryzacji wykazuje silną zależność Φ_{df} od stężenia DMU w roztworze i charakterystyczną dystrybucję stereodimerów. Na podstawie niezależnie zmierzonej stałej równowagi asocjacji warstwowej cząsteczek DMU w wodzie oraz w oparciu o uprzednio zaproponowany model dimeryzacji poprzez kompleksy asocjacyjne cząsteczek wzbudzonych do stanu singletowego (Lisewski & Wierzchowski, 1971, *Mol. Photochem.* **3**, 231 - 254), oceniono wydajność kwantową tworzenia dimerów w kompleksach DMU ($\Phi_{df}^{as}=0.028\pm0.004$) oraz udział wzbudzonego stanu tripletowego w dimeryzacji, przebiegającej w niższym zakresie badanych stężeń DMU. Przedyskutowano przyczyny ponad 2-3-krotnej różnicy w wydajnościach Φ_{df}^{as} dla DMU i 1,3-dwumetylotyminy na podstawie dystrybucji poszczególnych stereodimerów w fotoprodukcie oraz efektywności bezpromienistych procesów dezaktywacji wzbudzonego stanu singletowego.

Stwierdzono, że w warstwowych kompleksach asocjacyjnych DMU powstają przede wszystkim stereodimery *trans-syn* i *cis-syn*.

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RECENZJE KSIĄŻEK

Neiman M. B. and Gál D., THE KINETIC ISOTOPE METHOD AND ITS APPLICATION. Akadémiai Kiadó, Budapest, 1971; str. 309.

Badaniem szybkości reakcji i ich mechanizmu przy pełnym opisie procesów chemicznych zajmuje się dział chemii fizycznej zwany kinetyką. Problem szybkości i mechanizmów reakcji chemicznych jest ważny nie tylko z czysto teoretycznego punktu widzenia, ale też z punktu widzenia zastosowań praktycznych we wszystkich naukach przyrodniczych i różnych dziedzinach technicznych, które korzystają z chemii. Pierwsza metoda stężeń stacjonarnych Bodensteina otrzymała później interpretację matematyczną. Zakres tłumaczenia zjawisk kinetycznych rozszerzył uczony radziecki prof. Siemionow, opracowując metodę stężeń quasi stacjonarnych tłumaczącą mechanizmy rozgałęzionych reakcji łańcuchowych. Klasyczne te już dzisiaj metody nie umiały jednoznacznie ocenić mechanizmów reakcji chemicznych. Nie pozwalały bowiem precyzyjnie ustalać sekwencji powstawania struktur przejściowych, ich stężeń i przemian w toku reakcji. Wydaje się, że tę lukę w badaniu kinetyki procesów chemicznych wypełnia przedstawiona w monografii kinetyczna metoda izotopowa (Kinetic Isotope Method — KIM) opracowana przez znakomitego fizyko-chemika radzieckiego prof. M. B. Neimana, którą następnie rozwinął i wskazał praktyczne jej wykorzystanie D. Gál, profesor Uniwersytetu w Szeged na Węgrzech — zajmujący się radiochemią. KIM pozwala określić charakter i rząd reakcji chemicznej z uwzględnieniem udziału poszczególnych struktur pośrednich substratów biorących udział w reakcji, ich sekwencję oraz charakteryzuje rolę prekursorów w powstawaniu tych substancji przejściowych.

Pierwsze 4 rozdziały tej książki zawierają teoretyczny opis KIM w porównaniu z klasyczną kinetyką chemiczną, w następnych 6 rozdziałach czytelnik znajdzie pełny przegląd tej metody w praktycznych zastosowaniach w badaniu procesów oksydacyjnych, polimeryzacji i krakingu, katalizy heterogennej, izomeryzacji, estryfikacji i hydratacji. Ponadto została przedstawiona poważna rola KIM w badaniu zjawisk biologicznych i biochemicznych — procesów metabolicznych pod wpływem działania różnych czynników chemicznych i fizycznych.

Autorzy książki przekonują czytelnika, że w badaniach procesów chemicznych ogromną rolę odgrywa opracowywanie i stosowanie nowoczesnych metod fizycznych, które umożliwiają wnikanie w istotę zjawisk chemicznych. I tak np. znakowanie związków chemicznych izotopami trwałymi pozwala z powodzeniem zastosować w badaniach kinetyki procesów chemicznych — spektroskopię w podczerwieni, spektrometrię masową i elektronowy rezonans paramagnetyczny łącznie z KIM. Podwójne natomiast znakowanie obydwu węgli w cząsteczce ^{14}C i ^{13}C umożliwia wykrywanie ich jednocześnie metodą radioizotopową i za pomocą spektroskopii IR, co znacznie rozszerza zakres informacji o kinetycznym procesie reakcji chemicznych i biochemicznych. Autorzy wykazali za pomocą KIM, jak wielką rolę w każdym niemal procesie chemicznym odgrywa powstawanie chemicznie aktywnych produktów przejściowych — atomów, rodników i stosunkowo mało stabilnych związków, które stanowią dodatkowe źródło aktywnych struktur, w wyniku czego reakcje wykazują autokatalityczny charakter. W dodatku książki zawarty jest opis syntezy tych wszystkich związków znaczonych, które mogą być wykorzystane w badaniach metodą kinetyki izotopowej. Ponadto każdy rozdział uzupełnia bogate piśmiennictwo wynoszące od kilkunastu do ponad stu pozycji.

Jan Woźniak

IMMUNOPATHOLOGY. VIth International Symposium, Grindelwald (Switzerland) 1970, WHO Conference on Immunopathology of Viral Diseases. Schwalbe Co. Publishers, Basel and Stuttgart.

Omawiana książka jest zbiorem doniesień, wygłaszanych w czasie sympozjum. Dotyczyło ono przede wszystkim wirusowych schorzeń zwierząt. Użyte modelowe układy badawcze pozwoliły na uzyskanie ciekawych wyników i wysunięcie wniosków poszerzających wiedzę o mechanizmach procesów immunopatologicznych. Przykładem tego może być doniesienie A. L. Notkinsa. Wykazał on, że wirus dehydrogenazy kwasu mlekowego (LDV), podany myszom Balb/c przed przeszczepieniem im allogenicznego przeszczepu skóry z myszy C3H, statystycznie znamiennie opóźniał odrzucanie przeszczepu. Zakażenie tym wirusem myszy (Balb/c × ALN)F_1 hamowało powiększanie się śledziony w reakcji przeszczep przeciw gospodarzowi po wstrzyknięciu komórek śledziony z myszy Balb/c. D.D. Porter i A. E. Larsen przedstawili tezę, że morfologiczne (zapalenie kłębków nerkowych) i biochemiczne objawy choroby aleuckiej norek są uwarunkowane ciągłym współdziałaniem między stale obecnym wirusowym antygenem a swoistymi przeciwciałami, sam zaś proces replikacji wirusa wywołuje tylko nieznaczne uszkodzenia komórek. Kilka doniesień poświęcono immunopatologii zakażeń wywołanych przez wirusa limfocytowego zapalenia opon mózgowych (LCM). Wykazano, że zarówno dorosłe zakażone myszy, jak i myszy zakażone w okresie płodowym lub okołoporodowo były zdolne do humoralnej i komórkowej odpowiedzi immunologicznej. Wysunięto pogląd, że u myszy nosicieli wirusa wytwarza się stan tolerancji immunologicznej dotyczącej odporności komórkowej. Zakażenie wirusami LCM i polyoma myszy NZW i NZB prowadziło do uszkodzenia nerek, a mechanizm tego zjawiska dawał się wytłumaczyć wzrostem poziomu przeciwciał antyjądrowych i ich odkładaniem się w kłębkach nerkowych. Kompleksy immunologiczne antygen wirusowy + przeciwciało również odkładały się w kłębkach nerkowych. Samce myszy NZB były zdolne przekazywać swemu hybrydowemu potomstwu autoagresywną chorobę hemolityczną i własnego endogennego wirusa białaczki myszy, który prawdopodobnie brał udział w procesach autoimmunologicznych. Dwie ostatnie prace dotyczyły patologii człowieka. P.K. Russel w oparciu o hipotezę, że zespół wstrząsu przy dengue zachodzi po powtórnym zakażeniu heterologicznym typem wirusa dengue, przedstawił interesujący wykres mechanizmu tego zespołu. Przed wystąpieniem objawów zespołu wstrząsu szybko wzrastał poziom wiążących dopełniacz przeciwciał IgG. Zachodziło to w okresie wiremii. Powstawał kompleks antygen-przeciwciało, a o roli dopełniacza świadczył spadek poziomu składnika C'3. Powstający produkt rozpadu składnika C'3 miał właściwości anafilotoksyny odpowiedzialnej za objawy zespołu wstrząsu przy dengue. A. J. Zuckerman przytoczył szereg danych dotyczących odpowiedzi immunologicznej we wszczepionym zapaleniu wątroby, a immunopatogenezę uszkodzeń wątroby tłumaczył reakcjami alergicznymi i procesami autoimmunologicznymi.

Książka może zainteresować wirusologów, immunologów i patologów.

Michał Korbecki

INHERITED BLOOD CLOTTING DISORDERS. World Health Organization Technical Report Series no. 504; Geneva 1972; str. 48, cena S 1.00, fr. szw. 4.—

Kolejny, pięćset czwarty numer tego wydawnictwa zawiera sprawozdanie z zebrania grupy ekspertów Światowej Organizacji Zdrowia na temat dziedzicznych zaburzeń krzepnięcia krwi. Zebranie to odbyło się w Genewie w dniach 23—28 lipca 1971 r.

We wstępnej części sprawozdania zamieszczono schemat przebiegu krzepnięcia krwi, oparty na hipotezie Macfarlane'a z 1969 r. i uwzględniający kolejność udziału poszczególnych składników tego procesu. Scharakteryzowano poszczególne czynniki oraz zamieszczono zestawienie zaburzeń wynikających z ich braku.

Następnie omówiono genetyczne aspekty chorób krzepnięcia krwi ze specjalnym uwzględnieniem hemofilii.

Kolejne rozdziały przedstawiają kliniczne objawy zaburzeń krzepnięcia krwi i ich diagnozę; leczenie i prowadzenie chorych; oraz zagadnienia pozycji chorego w życiu rodzinnym i społecznym

(wyodrębniając zagadnienia dotyczące dzieciństwa, wieku młodzieńczego i dojrzałego, oraz specjalnych szkół dla chłopców ze skazą krwotoczną).

Omówiono następnie zagadnienia i obecny stan wiedzy na temat wykrywania osób będących nosicielami tych schorzeń i zagadnienia poradnictwa rodzinnego z punktu widzenia zagrożenia hemofilią.

Na zakończenie przedstawiono zalecenia grupy ekspertów dotyczące zadań służby zdrowia i kierunków dalszych badań w zakresie wykrywania nosicielstwa, diagnozy i leczenia zaburzeń krzepnięcia krwi. Podano również wzór Bayesa dla obliczenia prawdopodobieństwa występowania w danym przypadku heterozygotyczności w stosunku do hemofilii.

Irena Mochnacka

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