POLSKA AKADEMIA NAUK
KOMITET BIOCHEMICZNY I BIOFIZYCZNY

POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. XIV
No. 1

WARSZAWA 1967
PAŃSTWOWE WYDAWNICTWO NAUKOWE

ADDRESS

Krakowskie Przedmieście 26/28, Warszawa 64,
Poland

Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10
Nakł. 2226+154 egz. Ark. wyd. 18 ark. druk. 12+0,25+1,5 wkł.
Papier ilustr. druk. sat. kl. V, 70 g.
Oddano do składania 7.XI.66. Podpisano do druku 12.IV.67.
Druk ukończono w kwietniu 1967.
Zam. 752 T-74 Cena zł. 25.—
Warszawska Drukarnia Naukowa — Warszawa, Śniadeckich 8

This issue of Acta Biochimica Polonica, along with no. 4, vol. 13, is dedicated to Professor Dr. Józef Heller
and contains further papers submitted in his honour which, although they appear at a later date, are presented in the same spirit of esteem and affection

Editors

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

HENRYKA WIERZBICKA, A. B. LEGOCKI and J. PAWEŁKIEWICZ

DETERMINATION OF ACETATE AND PROPIONATE USING PROPIONATE (ACETATE) KINASE FROM *PROPIONIBACTERIUM SHERMANII*

Department of Biochemistry, College of Agriculture, Poznań

1. A very stable preparation of propionate(acetate) kinase from *P. shermanii* was obtained. The activity of the enzyme is not affected by 1 M concentration of NaCl, KCl and NH_4Cl. 2. Using the isolated kinase, an improved procedure for acetate and/or propionate determination in biological material was elaborated.

General properties of propionate(acetate) kinase (ATP : acetate phosphotransferase, EC 2.7.2.1) have been described by Pawełkiewicz & Legocki (1963), and Allen, Kellermeyer, Stjernholm & Wood (1964) isolated the enzyme in pure and homogeneous form. In the present work it was demonstrated that the enzyme can be utilized for determination of acetate and propionate in biological material. The elaborated procedure is an improved modification of the method of Rose, Grunberg-Manago, Korey & Ochoa (1954).

MATERIALS AND METHODS

Reagents. NAD and GSH (Sigma Chemical Co., St. Louis, Mo., U.S.A.); CoA (activity $\geqslant 90\%$) and tris(hydroxymethyl)aminomethane (Fluka A. G., Buchs S.G., Switzerland); ATP, disodium salt (Reanal, Budapest, Hungary); 2-mercaptoethanol (Light & Co. Ltd., Colnbrook, Bucks, England); $FeCl_3$ (BDH, Poole, Dorset, England). Other reagents were from Fabryka Odczynników Chemicznych (Gliwice, Poland).

Enzymes preparation. Propionibacterium shermanii was grown as described previously (Pawełkiewicz, Bartosiński & Walerych, 1961) in a medium containing acid and enzymic casein hydrolysate and glucose, then the cells were acetone-dried. Isolation of propionate(acetate) kinase was carried out at 0 - 4° in the presence of 5 mM-2-mercaptoethanol. Acetone-dried cells, 30 g., were extracted overnight with 300 ml. of 0.02 M-tris-HCl buffer, pH 7.8, then centrifuged for 15 min. at 20 000 *g* and 0°. The nucleic acids present in the supernatant were precipitated by adding 10 ml. of 20% streptomycin sulphate. The sediment was centrifuged off and the supernatant was fractionated with ammonium sulphate, a constant pH

value being maintained by adding 0.1 N-KOH. The enzyme activity was recovered in the 0.5 - 0.9 sat. fraction. The precipitate was collected by centrifuging, dissolved in 50 ml. of water and dialysed against water for 12 hr. The dialysis residue in the bag was concentrated to half its volume by a stream of air at 0°, and then freeze-dried. The yield was 300 - 400 mg. and the preparation contained 16 - 21 enzyme units per mg. protein. One kinase unit represents the amount of protein which catalyses the phosphorylation of 1 μmole of acetate per minute under the described conditions.

An enzymic preparation from rabbit heart muscle mitochondria as described by Korff (1954) was used.

Determination of propionate and/or acetate. This was based on the method of Rose *et al.* (1954). A sample containing 0.5 - 2.0 μmoles of the substrate(s) was incubated at 40° for 1 hr. in a medium containing in the final volume of 1 ml.: 50 μmoles of tris-HCl buffer, pH 8.1; 700 μmoles of hydroxylamine hydrochloride neutralized *ex tempore* with 700 μmoles of KOH; 10 μmoles of ATP (disodium salt); 10 μmoles of $MgCl_2$; and 8 - 16 units of the enzyme. The reaction was stopped by adding 1 ml. of 10% trichloroacetic acid. Then 4 ml. of 1.25% $FeCl_3$ solution in 1 N-HCl was added and the precipitated protein centrifuged off. In the supernatant the extinction of the coloured complex of acylhydroxamate and ferric ion was measured at 540 mμ in Spectronic 20 (Bausch & Lomb).

Determination of acetate. This was carried out according to Korff (1954). A neutralized sample containing 0.1 - 0.4 μmole of acetate was incubated for 30 min. at 37° in a mixture containing in a final volume of 1.5 ml.: 100 μmoles of GSH, 100 μg. CoA, 50 μmoles of tris-HCl buffer, pH 9.5, 10 μmoles of potassium malate, 500 μg. NAD, 2 μmoles of ATP (disodium salt), 6 μmoles of $MgCl_2$ and 0.05 ml. of mitochondrial extract from rabbit heart muscle. The reaction was stopped by adding 2 ml. of 0.5 M-phosphate buffer, pH 7.5. The amount of $NADH_2$ formed was estimated at 340 mμ in a Hilger H 700 spectrophotometer. A standard curve for acetate was prepared separately for each series of determinations.

Preparation of biological material for analysis. The culture of *P. shermanii* was neutralized with a saturated solution of Na_2CO_3, then 10 ml. of the bacterial suspension was centrifuged at 12 000 *g* for 15 min. The clear supernatant was diluted so as to obtain a concentration of acetate and propionate ranging from 0.2 to 1.0 μmole/ml. For determinations of the sum of acetate and propionate with the *P. shermanii* kinase the supernatant was diluted 10 - 25 times, and for the assays by the method of Korff (1954), 100 - 250 times.

Cow rumen contents, 10 g., was extracted with about 60 ml. of hot 0.005 N-KOH. The insoluble was centrifuged, washed with water and the combined supernatants were diluted for analysis to a volume of 100 ml. and 1000 ml., respectively.

RESULTS AND DISCUSSION

The common procedure for determination of acetate in the presence of other organic salts, is the use of acetate kinase and determination of the hydroxamate

formed from phosphoacetate and hydroxylamine. The kinases described so far are unstable and susceptible to inorganic salts, which makes necessary initial separation of the compounds to be determined. However, the kinase obtained from *P. shermanii* was found to be very stable and non-susceptible to inorganic salts, which permitted to elaborate a procedure for determination of acetate and/or propionate directly in biological material.

The results presented in Table 1 show that an increase in concentration of NaCl, KCl and NH_4Cl, even up to 1 M, virtually did not affect the accuracy of determinations made by the described method. In this respect the kinase from *P. shermanii* differs from acetate kinase of rumen bacteria (Campen & Matrone, 1964) and pigeon liver kinase which was applied by Soodak & Lipmann (1948) for determination of acetate.

The preparation obtained from 1 litre of an 8 - 10-day-old culture of *P. shermanii* (about 10 g. dry weight of cells) contained over 2000 kinase units; this amount was sufficient for performing 150 - 300 analytic determinations. The activity of the freeze-dried enzyme preparation was unchanged even after 6 months of storage at 4°, and the activity of the kinase in acetone-dried bacterial cells was practically unlimited.

Using standard acetate and propionate solution it was found that over the range of concentrations from 0.5 to 8.0 μmoles/ml., formation of the respective acylhydroxamates was linear (Fig. 1). Acetylhydroxamate gave slightly lower extinction values than propionylhydroxamate, these differences were, however, insignificant over the range 0.5 - 2.0 μmoles/ml. Therefore for determinations of the mixture of acetate and propionate not more than 2 μmoles of these compounds were taken.

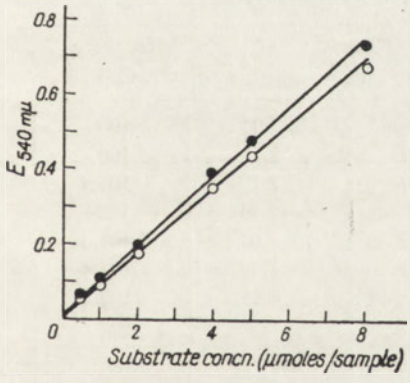


Fig. 1

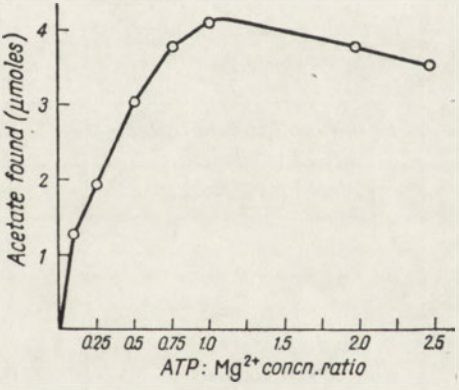


Fig. 2

Fig. 1. Determination of (○), acetate and (●), propionate in standard solution. The incubation mixture contained in 1 ml.: 16 units of propionate(acetate) kinase from *P. shermanii*; tris-HCl buffer, 50 μmoles, pH 8.1; hydroxylamine, 700 μmoles; ATP, 10 μmoles; $MgCl_2$, 10 μmoles and 0.5 - 8.0 μmoles of acetate or propionate. Incubation 1 hr. at 40°.

Fig. 2. The effect of the ATP : Mg^{2+} ratio on the activity of propionate(acetate) kinase from *P. shermanii*. The incubation mixture of pH 7.5 contained in 1 ml.: potassium acetate, 800 μmoles; tris-HCl buffer, 200 μmoles; hydroxylamine, 700 μmoles; $MgCl_2$, 10 μmoles; enzyme preparation, 60 μg.; and ATP 2.5 - 25 μmoles. Incubation: 2 min. at 40°.

Table 1

The effect of inorganic salts on propionate(acetate) kinase from P. shermanii *used in estimation of acetate and propionate in standard solution*

The incubation mixture contained in 1 ml.: 50 μmoles of tris-HCl buffer, pH 8.1; 700 μmoles of hydroxylamine; 10 μmoles of ATP; 10 μmoles of $MgCl_2$; 8 enzyme units and 2 μmoles of the acetate-propionate mixture. Incubation: 1 hr. at 40°. For details see Methods.

Salt added	Molar salt concn.					
	0.0	0.2	0.4	0.6	0.8	1.0
NaCl	2.00	2.05	2.10	1.95	2.00	1.95
KCl	2.00	2.10	2.05	2.10	2.00	1.95
NH_4Cl	2.00	1.95	2.00	1.95	1.90	1.95
K_2SO_4*	2.00	1.95	1.90	—	—	—

* Concn. of K_2SO_4 was, respectively, 0.25 and 0.50 M.

Table 2

The recovery of acetate added to the P. shermanii *culture medium or to cow rumen contents*

The bacterial culture was neutralized with Na_2CO_3, centrifuged, and the clear supernatant was appropriately diluted. The rumen contents were extracted with hot 0.005 N-KOH solution, and after centrifuging the supernatant was appropriately diluted. For the estimation, 16 units of propionate(acetate) kinase were applied.

Material	Acetate		Recovery (%)
	added	estimated	
P. shermanii culture containing 1.55 μmoles of acetate+propionate per sample	0.50	2.05	100
	0.50	2.05	100
	1.00	2.70	105.9
	1.00	2.60	102
	2.00	3.60	101.4
	2.00	3.60	101.4
	3.00	4.65	102.2
	3.00	4.65	102.2
Cow rumen contents containing 0.85 μmole of acetate+propionate per sample	0.50	1.35	100
	0.50	1.40	103.7
	1.00	1.90	102.7
	1.00	1.85	100
	2.00	2.85	100
	2.00	2.80	98.2
	3.00	3.80	98.7
	3.00	3.70	96.1

The kinase isolated from *P. shermanii* was specific toward propionate and acetate. Under the conditions of the experiment, formate, butyrate, malonate, succinate, α-oxoglutarate and malate did not interfere with the determinations.

The optimum ratio of ATP to Mg^{2+} ion for enzyme activity was 1 at ATP concentration of 10 mM. When this ratio amounted to 2 the kinase activity was lower by about 8% (Fig. 2).

The elaborated method was applied for direct determination in *P. shermanii* cultures and in cow rumen contents, of the sum of acetate and propionate as well as of the recovery of added acetate (Table 2). The average recovery in 16 determinations amounted to 100.9%. The calculated mean square error for the above method had a value of 2.6 whereas the maximum error of measurement was 4.6%.

To estimate acetate in the presence of varying amounts of propionate, the micromethod of Korff (1954) was applied and reliable results were obtained over a wide range of molar concentrations (Table 3).

The two analytical procedures when applied together, proved to be very useful for studying the course of acetate-propionate fermentation.

Table 3

Estimation of acetate in acetate+propionate mixtures of different concentration

The concentration of acetate+propionate did not exceed 2 μmoles/sample.

Acetate : propionate molar ratio	Recovery (%)	
	acetate+propionate [propionate(acetate) kinase method]	acetate [method of Korff (1954)]
1 : 1	97.5	100
	97.5	105
1 : 2	100	96.3
	100	92.6
1 : 3	97.5	105.1
	97.5	102.2
1 : 10	97.5	106.9
	97.5	103.4
2 : 1	97.5	100
	97.5	100
3 : 1	100	96.6
	97.5	100
10 : 1	97.5	102.5
	97.5	98.5

REFERENCES

Allen S.H.G., Kellermeyer R.W., Stjernholm R.L. & Wood H.G. (1964). *J. Bact.* **87**, 171.
Campen D.R. & Matrone G. (1964). *Biochim. Biophys. Acta* **85**, 410.
Korff R.W. (1954). *J. Biol. Chem.* **210**, 539.
Pawełkiewicz J. & Legocki A.B. (1963). *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **11**, 569.
Pawełkiewicz J., Bartosiński B. & Walerych W. (1961). *Acta Biochim. Polon.* **8**, 131.
Rose I.A., Grunberg-Manago M., Korey S.R. & Ochoa S. (1954). *J. Biol. Chem.* **211**, 737.
Soodak M. & Lipmann F. (1948). *Fed. Proc.* **7**, 190.

OZNACZANIE OCTANU I PROPIONIANU PRZY UŻYCIU KINAZY PROPIONIANOWEJ(OCTANOWEJ) Z *PROPIONIBACTERIUM SHERMANII*

Streszczenie

1. Otrzymano bardzo trwały preparat kinazy propionianowej(octanowej) z *P. shermanii*. NaCl, KCl i NH_4Cl w stężeniu 1 M nie miały wpływu na aktywność enzymu.

2. Opracowano ulepszoną metodę oznaczania octanu i propionianu lub ich mieszaniny w materiale biologicznym przy użyciu wyizolowanej kinazy.

Received 4 March, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

ZOFIA LASSOTA

NUCLEIC ACIDS IN NORMAL AND γ-RAYS IRRADIATED EGGS OF *BOMBYX MORI*

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

1. Normal eggs of *B. mori* contained at diapause about 0.2 mg. of DNA and 3 mg. of RNA per 1 g. The DNA content increased about sixfold on embryogenesis and RNA increased by about 70% but before hatching decreased back to diapausal level. 2. The effects of γ-rays given at diapause, were visible at postdiapausal period only; 5 kr. resulted in delayed DNA synthesis and reduced by 15% the hatching; 20 kr. delayed the synthesis of DNA, damaged the synthesis of RNA and inhibited hatching completely; 200 kr. stopped the synthesis of DNA and induced the break-down of RNA. 3. The eggs were less susceptible to 20 kr. when irradiated after the peak of RNA synthesis had been attained. 4. The damage of gastrular RNA synthesis is suggested as a probable cause of injured embryogenesis in 20 kr. treated eggs.

It was previously reported (Lassota, 1963, 1965a, 1965b) that high doses of γ-rays, which inhibited completely the hatching of larvae when given on *B. mori* eggs at diapause, did not influence the end products of nitrogen metabolism of the eggs and blocked the postdiapausal respiration only at a rate characteristic for normal gastrula. The alteration of nucleic acid metabolism, especially of DNA, seemed the most probable event preceding the delayed mitotic death of irradiated embryo.

In this paper the results of investigation of nucleic acids in normal and irradiated eggs of *B. mori* are presented. The development of diapausing eggs of *B. mori* from the same crop is well synchronized: during diapause the development remains arrested at blastular stage A (Mikhailov, 1950) and the postdiapausal embryogenesis, regulated by gradual increase of storage temperature to occur within about 4 weeks results in almost simultaneous hatching of larvae from more than 80% of eggs. Thus the results of quantitative estimations made in large portions of simultaneously incubated eggs, presented here, can be considered as an average approximating the values for an individual egg at given stage of development.

MATERIAL AND METHODS

The eggs of monovoltine "warska" race of *Bombyx mori* were purchased from the industrial culture Milanówek. One batch from 1962 and two batches from 1963 were investigated. The eggs collected in early autumn were stored for 3 - 4 months at 15° and thereafter for at least 3 months at 4°. The postdiapausal development was promoted in spring by raising gradually the storage temperature to, and maintaining it at, 25°. The irradiated eggs and the non-irradiated control samples from the same batch were stored and allowed to develop simultaneously in identical conditions.

Irradiation. Irradiations were performed at the beginning of storage at 4° (mid-diapause) or during postdiapausal development as indicated in text. Single doses of 5 kr., 20 kr. or 200 kr. of γ-rays were delivered from ^{60}Co source (45 curie) at a rate of about 200 kr./min.

Extraction. Whole eggs, 500 mg., homogenized in a chilled glass homogenizer of Potter-Elvehjem type, with 10 ml. of ice-cold chloroform - acetone mixture (1:5, v/v) were delipidated after Niemierko, Niemierko & Włodawer (1952). Three extractions with 10 ml. portions of chloroform - acetone mixture at 0°, were followed by two extractions with 10 ml. portions of acetone at room temperature and subsequently by three extractions with 5 ml. portions of boiling ethanol - ether mixture (3 : 1, v/v). The delipidated material washed once with 10 ml. ice-cold acetone, was extracted twice at 0° with 10 ml. portions of ice-cold 0.3 N-$HClO_4$. The pooled acid extracts represented the acid-soluble fraction, containing nucleotides of the eggs.

The residue was hydrolysed according to Schneider (1945) and Schneider, Hogeboom & Ross (1950) with 5 ml. of 0.5 N-$HClO_4$ at 90° for 15 min. and centrifuged. The solid residue was washed with 5 ml. of 0.5 N-$HClO_4$. The combined supernatants represented the nucleic acid fraction of the eggs. The acid-soluble as well as the nucleic acid fractions were deproteinized according to Sevag, Lockman & Smolens (1938).

Standard solutions. Yeast RNA (L. Light and Co. Ltd., Colnbrook, Bucks, England) purified by Woodward's modification (1944) of Kunitz's method (1940) and herring sperm DNA, commercial (L. Light and Co.) were used for standard solutions. The solutions were prepared by hydrolysis of known amounts of RNA or DNA in 0.5 N-$HClO_4$ for 15 min. at 90°, and dilution of resulting hydrolysates with 0.5 N-$HClO_4$ to obtain concentrations of 20 - 200 μg./ml. The ε(P) of standard hydrolysates was controlled and values of 10 380 for RNA and 8 640 for DNA were found. These standard solutions were used as reference in spectrophotometric and colorimetric estimations of nucleic acids in egg extracts.

Analytical methods. The UV absorption spectra were read on a Unicam SP 500 spectrophotometer, against appropriate blanks. Deoxyribose was determined by Burton's (1956) modification of diphenylamine method. Ribose was determined by orcinol method of Mejbaum (1939) and the results were corrected for deoxyribose present in extracts, according to Schneider (1945). Phosphorus was determined by Fiske-Subbarow's method.

RESULTS

Normal eggs

The nucleic acid content found in diapausing eggs is given in Table 1 (control values). The changes occurring on postdiapausal development are shown in Tables 2, 3 and 4. The amounts of nucleic acids found per weight unit of the eggs in batches from 1963 (Tables 3 and 4) were higher than those determined in the batch from 1962 (Table 2); however, the changes occurring on development were the same in both: the DNA content of the eggs rose about 6-fold; the increase in RNA was relatively small (maximally by about 70%); the RNA/DNA ratio dropped from 16 to 3. Shortly before hatching the RNA content decreased back to the initial level.

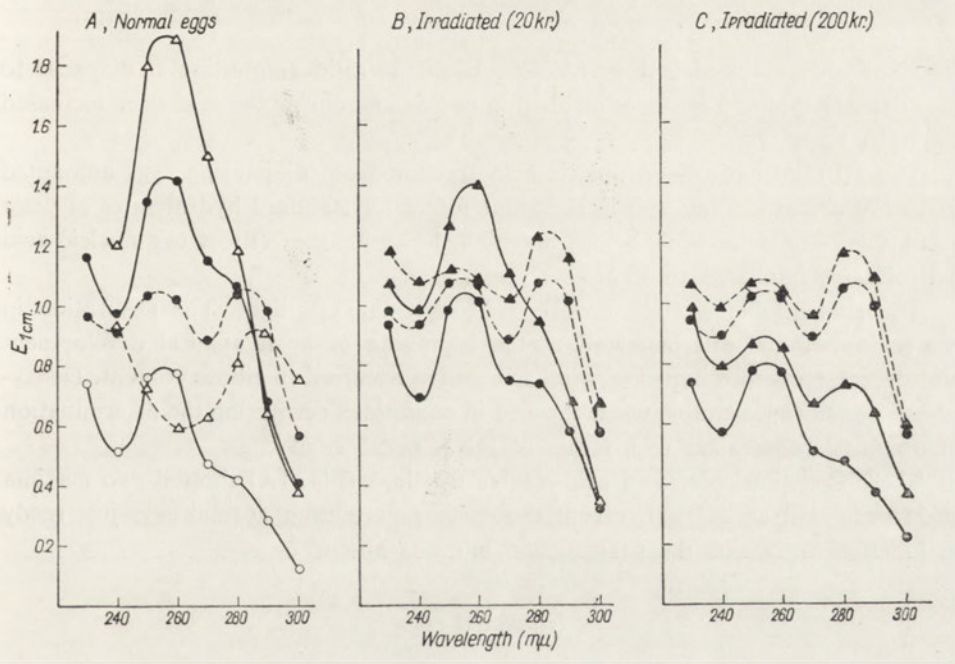


Fig. 1. Ultraviolet absorption spectra of (——), nucleic acids, and (– – –), acid-soluble fractions. *A*, Non-irradiated eggs; *B*, eggs irradiated with 20 kr. at mid-diapause; *C*, eggs irradiated with 200 kr. at mid-diapause, and investigated: (○), at diapause; (●), 6 days before hatching (for *B* and *C*, before the hatching of controls); (△), 1 day before hatching; (▲), 1 day after the hatching of controls. In all diagrams are presented the values of absorption corresponding to the same quantitites of eggs.

The absorption at 260 mμ of deproteinized hydrolysates of the nucleic acid fraction increased gradually on development parallelling the trend of changes found by colorimetric methods. Shortly before hatching, the absorption values were, however, by about 70% higher than the values corresponding to the sum of RNA and DNA estimated colorimetrically (Table 3). The absorption spectra of these

Table 1

The nucleic acids of normal and γ-irradiated eggs of Bombyx mori *at mid-diapause (batch* 1962)

The absorption of nucleic acid fraction at 260 mμ was expressed in mg. of nucleic acids. Deoxyribose was determined after Burton (1956), ribose after Mejbaum (1939), the results being corrected for deoxyribose content according to Schneider (1945). All samples to be analysed were deproteinized after Sevag *et al.* (1938).

Irradiation dose (kr.)	No. of samples	mg./g. of whole eggs		
		DNA+RNA (E_{260})	RNA (ribose)	DNA (deoxyribose)
None (control)	8	1.93±0.38	2.92±0.33	0.27±0.06
5	3	1.74±0.47	2.95±0.33	0.23±0.05
20	5	1.77±0.35	2.97±0.45	0.18±0.05

fractions are presented in Fig. 1A. The E_{260}/E_{280} ratio amounting at diapause to 1.86 decreased after 3 weeks of incubation to 1.34 and during the next week increased again to 1.59.

The ε(P) of hydrolysed nucleic acid fraction from diapausing eggs amounted to 10 270 corresponding well to the value found for standard hydrolysates of yeast RNA (10 380). One week before hatching, however, the ε(P) of egg nucleic acid hydrolysates increased to 15 210.

The acid-soluble fraction absorbed strongly the UV light. The absorption in the region of 260 mμ increased at the beginning of postdiapausal development and decreased before hatching. Similar changes occurred in ribose content. Deoxyribose was not measurable when checked in conditions permitting the determination of quantities equivalent to 0.1 mg. of DNA per 1 g. of eggs.

The absorption spectra of acid-soluble fractions (Fig. 1A) showed two maxima at 255 mμ and at 285 mμ, except the curve representing normal eggs just ready to hatch, from which the peak at 255 mμ was absent.

Irradiated eggs

The nucleic acids of the eggs irradiated at mid-diapause with 5 kr. and 20 kr. of γ-rays did not change significantly as long as the development was arrested (Table 1). The effects of irradiation became evident when the storage temperature was raised to the level allowing postdiapausal embryogenesis. In the eggs irradiated with 5 kr. (Table 2) the synthesis of DNA and RNA occurred, the increase in DNA was, however, revealed a few days later than in the simultaneously incubated control. The hatching of larvae from 5 kr. treated eggs was delayed by 3 days and amounted to 85% of incubated eggs.

In 20 kr. treated eggs the synthesis of both DNA and RNA did not follow normal patterns (Table 3). The increase in DNA was retarded so that the amount found in these eggs after 4 weeks of incubation (0.8 mg./g.) was equal to that found

in simultaneously incubated control already after 3 weeks. This difference, however, could not be interpreted in terms of a simple delay in normal development. When the irradiated and normal eggs, each containing the same amounts of DNA, were compared differences indicating general disharmony in nucleic acid synthesis were evident: in irradiated eggs the RNA content, the absorption at 260 mμ of hydrolysed nucleic acid fraction and the ribose content of acid-soluble fraction were substantially lower.

In 200 kr. treated eggs (Table 4) the DNA content remained during 4 weeks of postdiapausal incubation at the level found at diapause. The RNA content of these eggs decreased below the initial level after 3 weeks of incubation and this decrease was accompanied by augmentation of ribose in acid-soluble fraction. The microscopical examination proved that at this period first signs of disintegration of embryonal tissue were visible.

Neither at diapause nor during postdiapausal incubation measurable amounts of deoxyribose were found in acid-soluble fractions from both 20 kr. and 200 kr. treated eggs.

The UV absorption spectra of hydrolysed nucleic acid fractions from irradiated eggs are presented in Fig. 1B and C. The differences in E_{260}/E_{280} ratio between 20 kr. treated and control eggs were evident only during the last week of incubation, the ratio increasing at this period to 1.47 in irradiated and to 1.59 in control eggs. The curve representing 200 kr. treated eggs differed markedly from control. The E_{260}/E_{280} ratio after 3 weeks of incubation remained in irradiated eggs at the level observed in diapausing control eggs and during the last week it dropped sharply to 1.18.

The ε(P) of hydrolysed nucleic acid fraction from 20 kr. treated eggs amounted to 16 710 and 16 450 after 3 and 4 weeks of incubation, respectively, thus slightly above the value characteristic for normal eggs one week before hatching [ε(P) 15 210]. The ε(P) values found in nucleic acid fraction from 200 kr. treated eggs were abnormally high and during the last week of incubation rose from 18 030 to 30 140.

The absorption maximum at 255 mμ of the acid-soluble fractions which in normal eggs vanished after 4 weeks of incubation was still present at this period in 20 kr. as well as in 200 kr. treated eggs.

In the eggs irradiated at mid-diapause, the long period of metabolic arrest and storage at low temperature following irradiation, could be anticipated to modify the effects of irradiation. Therefore the effects upon the nucleic acids of eggs, of 20 kr. of γ-rays applied at mid-diapause or at various stages of postdiapausal incubation were compared. The results expressed as percentages of diapausal values are presented in Table 5. No marked differences were observed between the eggs irradiated at mid-diapause and those treated during the first 18 days of postdiapausal incubation, thus still before the DNA content attained the value of 0.8 mg./g. of eggs. In those eggs further synthesis of DNA was delayed, RNA never attained the normal maximum and complete inhibition of hatching resulted. When, however, the irradiation was applied on the eggs in which more than 0.8 mg. of DNA

Table 2

The influence of irradiation with 5 kr. at mid-diapause on the nucleic acids in B. mori *eggs during postdiapausal incubation (batch 1962)*

Procedure as given in Table 1. Control and irradiated eggs were incubated simultaneously in identical conditions.

Investigated at		Found in 1 g. of whole eggs							
		Control eggs				Irradiated eggs			
day before hatching in control	storage temperature (°C)	RNA +DNA (mg.) (E_{260})	RNA (mg.) (ribose)	DNA (mg.) (deoxy-ribose)	RNA/DNA ratio	RNA +DNA (mg.) (E_{260})	RNA (mg.) (ribose)	DNA (mg.) (deoxy-ribose)	RNA/DNA ratio
25	10°	2.0	2.7	0.2	14	2.0	2.7	0.2	14
18	13°	2.6	3.1	0.3	10	2.6	3.2	0.2	16
11	15°	3.3	3.8	0.6	6	2.2	3.1	0.3	10
5	20°	4.8	4.4	1.3	3	3.9	4.2	1.0	4
2 days after hatching in control	25°	hatching from 95% of incubated eggs				6.8	3.2	1.3	3

Table 3

The influence of irradiation with 20 *kr. at mid-diapause on the nucleic acids of* B. mori *eggs during postdiapausal incubation (batch A-*1963*)*

Procedure as given in Table 1. Acid-soluble fraction was deproteinized according to Sevag *et al.* (1938). Control and irradiated samples were incubated simultaneously in identical conditions.

Investigated at		Found in 1 g. of whole eggs											
		Control eggs						Irradiated eggs					
		nucleic acid fraction				acid-soluble fraction		nucleic acid fraction				acid-soluble fraction	
day before hatching in control	storage temperature (°C)	RNA +DNA (mg.) (E_{260})	RNA (mg.) (ribose)	DNA (mg.) (deoxy-ribose)	RNA/ DNA ratio	ribose nucleo-tides (mg.) (ribose)	$E^{1\ cm.}_{260}$	RNA +DNA (mg.) (E_{260})	RNA (mg.) (ribose)	DNA (mg.) (deoxy-ribose)	RNA/ DNA ratio	ribose nucleo-tides (mg.) (ribose)	$E^{1\ cm.}_{260}$
28	10°	3.1	3.3	0.2	16	4.4	200	3.0	3.2	0.2	16	4.2	200
21	13°	3.9	4.1	0.3	14	4.9	230	3.8	3.6	0.3	12	5.6	240
14	15°	4.8	3.8	0.4	9	5.0	280	3.6	3.6	0.3	12	4.0	270
8	20°	5.2	5.5	0.8	7	4.5	240	4.0	4.5	0.4	11	4.2	230
6	20°	5.9	4.0	1.0	4	3.7	250	4.0	3.4	0.4	8	3.3	250
1	25°	7.5	3.3	1.3	3	3.2	170	4.7	3.4	0.8	4	3.2	220
1 day after hatching in control	25°	hatching from 95% of incubated eggs						5.6	3.6	1.2	3	3.5	260
								no hatching during next week of incubation at 25°					

Table 4

The influence of irradiation with 200 *kr. at mid-diapause on the nucleic acids of* B. mori *eggs during postdiapausal incubation (batch B-*1963*)*

Procedure as given in Table 1. Acid soluble-fraction was deproteinized according to Sevag *et al.* (1938). Control and irradiated eggs were incubated simultaneously in identical conditions.

Investigated at		Found in 1 g. of whole eggs											
		Control eggs						Irradiated eggs					
		nucleic acid fraction				acid-soluble fraction		nucleic acid fraction				acid-soluble fraction	
day before hatching in control	storage temperature (°C)	RNA +DNA (mg.) (E_{260})	RNA (mg.) (ribose)	DNA (mg.) (deoxy-ribose)	RNA/ DNA ratio	ribose nucleo-tides (mg.)	$E^{1\ cm.}_{260}$	RNA +DNA (mg.) (E_{260})	RNA (mg.) (ribose)	DNA (mg.) (deoxy-ribose)	RNA/ DNA ratio	ribose nucleo-tides (mg.)	$E^{1\ cm.}_{260}$
27	10-12°	3.0	3.2	0.2	16	4.0	200	2.8	3.0	0.2	15	4.0	210
20	13-14°	3.4	3.7	0.2	18	4.6	230	3.0	3.7	0.2	18	4.8	230
13	16°	4.2	3.6	0.4	8	4.5	220	2.9	3.1	0.2	16	2.4	230
6	20°	5.9	4.0	1.0	4	3.7	250	3.3	2.6	0.2	13	3.4	230
1	25°	7.4	3.4	1.2	3	3.0	180						
1 day after hatching in control	25°	hatching from 95% of incubated eggs						3.5	2.7	0.2	13	3.7	250
								no hatching during next week of incubation at 25°					

Table 5

The influence of irradiation with 20 *kr. applied at various stages of development of* B. mori *eggs on their nucleic acids (batch B-*1963*)*

Results expressed as percentages of diapausal values found in nucleic acid fractions. Analytical procedure as given in Table 1. All investigated samples were incubated simultaneously in identical conditions.

Investigated at		Control eggs			Eggs irradiated with 20 kr. at													
					mid-diapause			postdiapausal incubation, before hatching in control										
								15 days			12 days			10 days			7 days	
day before hatching in control	storage temperature (°C)	DNA	RNA	E_{260}	DNA	RNA	E_{260}	DNA	RNA	E_{260}	DNA	RNA	E_{260}	DNA	RNA	E_{260}	DNA	RNA	E_{260}
18	13°	150	115	130				150	115	130	150	115	130	150	115	130	150	115	130
14	15°	200	100	155	150	110	120				200	100	155	200	100	155	200	100	155
11	15°	300	140	165										300	140	165	300	140	165
8	20°	400	170	170	150	130	135	200	150	150							400	170	170
6-5	25°	650	160	240	200	100	135				500	150	210	350	155	165	600	175	245
1	25°	650	100	240	400	100	150	400	110	160				550	105	190			
1-2 days after hatching in control	25°	hatched			600	110	190				600	140	325				800	190	480
7 days after hatching in control	25°				no hatching			no hatching			no hatching			no hatching			hatching from 10% of incubated eggs		

per 1 g. of eggs had accumulated and the maximum RNA level was already attained, the hatching of larvae occurred, albeit it was delayed and reduced to about 10% of examined eggs.

DISCUSSION

Normal eggs

Only few data on the nucleic acid content of *B. mori* eggs are available. According to Chino (1956) in freshly laid eggs the phosphorus of nucleic acids amounted to 0.08 μg. per one egg and DNA accounted for 1/4 of this value. Shortly before hatching Chino has found 0.10 μg. of DNA-P and 0.25 μg. of RNA-P per one egg. Niemierko, Włodawer & Wojtczak (1956) have investigated the eggs of *B. mori* at diapause and stated 0.18 μg. of nucleic acid phosphorus per one egg, DNA accounting for less than 1/10 of this value. The results presented here, calculated per one egg, correspond to 0.016 μg. of DNA-P and 0.19 μg. of RNA-P at diapause and to 0.09 μg. and 0.25 μg., respectively, at the end of postdiapausal embryogenesis. Despite different methods of analysis used, the presented data on diapausal and final DNA and RNA content of the eggs reasonably agree with those reported by Chino as well as by Niemierko *et al.*

The comparison of discussed data indicates that the DNA content of freshly laid eggs does not change till the end of diapause and the net synthesis of DNA begins only at late blastula stage. A similar situation reported by Baltus & Brachet (1962) for amphibian eggs was interpreted as being due to the so-called "DNA cytoplasmatic reserve" sufficient to provide for the synthesis of nuclear DNA till the late blastula stage. On the contrary, the prediapausal synthesis of RNA in *B. mori* eggs is considerable, the amount of RNA in diapausing eggs being twice as great as in freshly laid ones. The accumulation during this period of ribosomal RNA and perhaps of long-living messenger RNA stored during diapause and utilized in the first steps of postdiapausal development is suggested. The existence of stocks of these compounds for future use was repeatedly reported for eggs of sea urchin (Gross, Spindel & Cousineau, 1963; Gross, Malkin & Moyer, 1964; Brachet, Denis & de Vitry, 1964; Nemer, 1964; Gross, Kraemer & Malkin, 1965), frog (Brown & Littna, 1964; Decroly, Cape & Brachet, 1964) and fish (Spirin, Belitsina & Aitkhozin, 1964) at various stages of early embryogenesis. The postdiapausal synthesis of RNA might be connected with gastrular differentiation and later stages of embryogenesis.

The discrepancy between the values of UV absorption and the results of colorimetric analysis of nucleic acids remains unexplained. At an advanced stage of embryogenesis, the ε(P) values of the hydrolysed nucleic acid fraction were markedly higher than those of yeast RNA (10 380) as well as of sperm DNA (8 640), indicating probably some structural specificity of nucleic acids of *B. mori* embryo. Levenbook, Travaglini & Schultz (1958) reported that in alkaline hydrolysates of nucleic acids from *Drosophila* eggs the ε(P) value was higher than that calculated on the basis of standard nucleotides.

The E_{260}/E_{280} ratio of the nucleic acid fraction changes on embryogenesis, despite the identical methods of isolation, reflecting perhaps some changes in nucleoproteins occurring on development.

The synthesis of nucleic acids was reflected in the changes in acid-soluble fraction. From the two maxima observed in UV absorption spectra of this fraction, that at 285 mμ was due to the presence of uric acid, already reported and discussed previously (Lassota, 1965a), and that at 255 mμ would represent the pool of nucleic acid precursors. The changes in UV absorption and in ribose content of the acid-soluble fraction (see control values in Tables 3 and 4) indicate that the pool of ribose-containing precursors initially rose, preceding slightly the synthesis of RNA. Thereafter, however, this pool decreased although the RNA content also decreased, and solely the DNA synthesis proceeded. At the end of embryogenesis the peak at 255 mμ vanished, reflecting the exhaustion of precursor pool (cf. Fig. 1A). This observation can be explained only if the hypothesis of Brachet (1965) that ribonucleosides can be used as DNA precursors, is accepted. It should be noted that in chinese tussur moth the physiological decrease in nucleic acid content does not cause an increase in pentose content of the acid-soluble fraction (Heller & Jeżewska, 1958).

The nucleic acid content of the eggs of at least three species of insects diapausing at the egg-stage were investigated, namely *Bombyx mori* (Chino, 1956), *Locusta migratoria* L. (Moureaux, 1963) and *Melanoplus differentialis* (Lu & Bodine, 1953). During diapause the DNA content of the studied eggs remained unchanged whereas the RNA content, which in *B. mori* eggs was constant too, decreased slightly in the eggs of both *Melanoplus* and *Locusta* (in the latter, however, only when diapausing at 5°). During postdiapausal development the DNA content of *B. mori* eggs increased 5-6-fold, while in *Melanoplus* and *Locusta* eggs 22- and 19-fold increase, respectively, was reported. The relatively small (less than twofold) increase in RNA found during this period in *B. mori* and *Melanoplus* eggs is contrasted with an about 16-fold increase reported for *Locusta* eggs. The RNA/DNA ratio, which in *Bombyx mori* and *Melanoplus* is greater than 1 during the whole life of egg, was found in *Locusta* eggs to decrease at diapause below 1 and to attain this value only just before hatching.

Irradiated eggs

After irradiation with 200 kr. the synthesis of DNA in *B. mori* eggs was completely inhibited. According to Alexander & Bacq (1960) the doses of this order suffice to produce *in vivo* main-chain scissions of DNA molecules. Thus the inhibition of synthesis of DNA in *B. mori* eggs can be attributed to the severe damage of molecules to be replicated.

The total inhibition of DNA synthesis and thus of mitotic activity in 200 kr. irradiated eggs did not immediately result in drastic break-down processes. The decrease in RNA accompanied by the accumulation of low molecular degradation

products as well as the striking rise in ε(P) and sharp decrease in E_{250}/E_{280} ratio of the nucleic acid fraction was revealed only after 3 weeks of incubation at elevated temperature.

It should be noted that in the eggs treated at diapause with 200 kr. the respiration is blocked at a low level but not arrested, no ammonia is released (Lassota, 1965b) and no accumulation of uric acid occurs (unpublished results) at least during 10 days of postdiapausal incubation at 25°. These observations agree well with the morphological ones (Tahmisian, 1949) made in the 200 kr. treated eggs of *Melanoplus*, and suggest that after this dose the insect's embryos are rather blocked than dead not only at diapause but also during at least 10 days of postdiapausal incubation. Thus the radioresistance of embryonal cells in which no mitoses occur is in insects as high as that of non-regenerating tissues of adults.

The effects of 20 kr. dose of γ-rays were quite different. Neither the DNA synthesis nor the synthesis of RNA were inhibited. The increase in the activity of nucleases, almost always observed in irradiated mammals (Goutier, 1961), was not checked in *B. mori* eggs; however, if present, it was efficiently compensated by the synthesis of new molecules and did not lead to the accumulation of low molecular degradation products. The synthesis of DNA, although delayed, continued until the level characteristic for the last stage of embryogenesis was attained. Thus the enzyme system responsible for DNA synthesis resisted this dose of γ-rays. The radiation damage of DNA molecules did not prevent their replication. This is in agreement with the known fact that the denatured DNA may be a good primer in the polymerase system of Kornberg (see Grunberg-Manago, 1963). The changes induced in DNA molecules by irradiation may, however, influence the DNA-dependent synthesis of RNA. The formation of abnormal mRNA and perhaps partial inhibition of its synthesis as well as of polysome formation may result. The effects of these events may be, however, reflected in the embryonal development at gastrula stage. Beginning with this stage the synthesis of new mRNA and/or ribosomal RNA is a prerequisite of embryonal differentiation in the eggs of sea urchin (Gross *et al.*, 1964; Nemer, 1964), frog (Brown & Littna, 1964; Deuchar & Bristow, 1965) and fish (Spirin *et al.*, 1964). Indeed in *B. mori* eggs treated with 20 kr. of γ-rays the maximum RNA level attained during postdiapausal incubation was by 20% lower than the maximum reached normally, indicating partial inhibition of gastrular RNA synthesis. This observation is consistent with the earlier one that the postdiapausal respiration of these eggs is blocked at the level characteristic for normal gastrula (Lassota, 1965b). The role of the injury of gastrular RNA synthesis in the delayed radiation death of *B. mori* embryo is supported by the results presented in Table 5. The same dose of 20 kr. of γ-rays was less deleterious when delivered on the eggs in which the maximum RNA content had been already attained, so that even the hatching of larvae occurred.

The disharmony between the DNA and RNA synthesis due to radiation was reflected in the UV absorption spectra of the nucleic acid fraction observed at the end of postdiapausal incubation. The absorption at 260 mμ of this fraction from 20 kr. treated eggs was markedly lower, although the level of nucleic acids was

at this moment the same as in normal eggs ready to hatch. The E_{260}/E_{280} ratio was at this period also lower than in normal eggs.

There are several analogies between the observed effects of γ-rays on the embryonal development and those of actinomycin D. This last is known to inhibit the genetic transcription by the inhibition of DNA-dependent RNA synthesis (Merits, 1963); its influence on DNA replication is not very effective since it combines more strongly with double-stranded DNA than with the replicating single-stranded molecule (Kahan, Kahan & Hurwitz, 1963). However, the inhibition of DNA replication occurs, but higher concentrations of actinomycin are needed (Hurwitz, Furth, Malamy & Alexander, 1962). When relatively high doses of actinomycin were injected into the blastocoele of *Rana pipiens* blastulae the development was arrested at this stage (Wallace & Elsdale, 1963). The external application of actinomycin D on the blastulae of *Bufo arenarum* permitted numerous and apparently normal mitoses to occur until the rudimentary nervous system began to form. In the arrested gastrulae the synthesis of nuclear RNA was inhibited and the underdevelopment of embryonal nervous system resulted (Brachet *et al.*, 1964). It should be noted that some of our results (Lassota & Grzelak, 1966) indicate abnormal neurulation in 20 kr. treated eggs of *B. mori*. So acetylcholine appearing at the end of embryogenesis amounted in normal eggs to about 80 μg./g. of eggs, whereas in irradiated eggs (investigated when the postdiapausal synthesis of DNA has been already accomplished) less than a half of this amount was found. The observations presented here and the analogy between the effects of γ-rays and of actinomycin would suggest that the first metabolic effect of 20 kr. dose may be the injury of transcription process. The replication of DNA and the mechanism of protein synthesis, except the mRNA formation, seems to be less important in the radiation injury.

REFERENCES

Alexander P. & Bacq Z.M. (1960). *Symposium on initial effects of ionizing radiation on cells, Moscow.* Academic Press.
Baltus E. & Brachet J. (1962). *Biochim. Biophys. Acta* **61**, 157.
Brachet J. (1965). *Progr. Biophys. Mol. Biol.* **15**, 99.
Brachet J., Denis H. & de Vitry F. (1964). *Developm. Biol.* **9**, 398.
Brown D.D. & Littna E. (1964). *J. Mol. Biol.* **8**, 669.
Burton K. (1956). *Biochem. J.* **62**, 315.
Chino H. (1956). *Embryologia* **3**, 167.
Decroly M., Cape M. & Brachet J. (1964). *Biochim. Biophys. Acta* **87**, 34.
Deuchar E.M. & Bristow D.A. (1965). *Nature* **205**, 1321.
Goutier R. (1961). *Progr. Biophys. Mol. Biol.* **11**, 53.
Gross P.R., Spindel W. & Cousineau G.H. (1963). *Biochim. Biophys. Res. Commun.* **13**, 405.
Gross P.R., Malkin L.J. & Moyer W.A. (1964). *Proc. Natl. Acad. Sci.* **51**, 407.
Gross P.R., Kraemer K. & Malkin L.J. (1965). *Biochim. Biophys. Res. Commun.* **18**, 569.
Grunberg-Manago M. (1963). *Progr. Biophys. Mol. Biol.* **13**, 175.
Heller J. & Jeżewska M. (1958). *Acta Biochim. Polon.* **5**, 3.
Hurwitz J., Furth J.J., Malamy M. & Alexander M. (1962). *Proc. Natl. Acad. Sci.* **48**, 1222.
Kahan E., Kahan F.M. & Hurwitz J. (1963). *J. Biol. Chem.* **238**, 2491.

Kunitz H. (1940). *J. Gen. Physiol.* **24**, 15.
Lassota Z. (1963). *Acta Biochim. Polon.* **10**, 379.
Lassota Z. (1965a). *Acta Biochim. Polon.* **12**, 271.
Lassota Z. (1965b). *Acta Biochim. Polon.* **12**, 369.
Lassota Z. & Grzelak K. (1966). *Bull. Acad. Polon. Sci., Ser. Biol.* **14**, 391.
Levenbook L., Travaglini E.C. & Schultz J. (1958). *Exptl. Cell. Res.* **15**, 43.
Lu K.H. & Bodine J.H. (1953). *Physiol. Zool.* **26**, 242.
Mejbaum W. (1939). *Z. physiol. Chem.* **258**, 117.
Mikhailov E.N. (1950). *Sholkovodstvo.* Gos. Isdat. Selskokhoz. Lit., Moskva.
Merits I. (1963). *Biochem. Biophys. Res. Commun.* **10**, 254.
Moureaux T. (1963). *Ann Biol. anim. Biochem. Biophys.* **3**, 33.
Nemer M. (1964). *Natl. Canc. Inst. Monograph.* No. 13, 141.
Niemierko W., Niemierko S. & Włodawer P. (1952). *Acta Biol. Exper.* **16**, 247.
Niemierko W., Włodawer P. & Wojtczak A.F. (1956). *Acta Biol. Exper.* **17**, 255.
Schneider W.C. (1945). *J. Biol. Chem.* **161**, 293.
Schneider W.C., Hogeboom G.H. & Ross H.E. (1950). *J. Natl. Canc. Inst.* **10**, 977.
Sevag M.G., Lockman D.B. & Smolens J. (1938). *J. Biol. Chem.* **124**, 425.
Spirin A.S., Belitsina N.V. & Aitkhozin M.A. (1964). *Zhurn. Obshch. Biol.* **25**, 312.
Tahmisian T.N. (1949). *J. Exptl. Zool.* **112**, 449.
Wallace H. & Elsdale T.R. (1963). *Acta Embryol. Morphol. Exptl.* **6**, 275.
Woodward G.E. (1944). *J. Biol. Chem.* **156**, 143.

KWASY NUKLEINOWE W JAJACH *B. MORI* W CZASIE NORMALNEGO ROZWOJU I PO NAŚWIETLANIU PROMIENIAMI γ

Streszczenie

1. Normalne jaja *B. mori* w diapauzie zawierają ok. 0,2 mg DNA i ok. 3 mg RNA na 1 g jaj. W czasie rozwoju embrionalnego zawartość DNA rośnie sześciokrotnie, a zawartość RNA rośnie o ok. 70% po czym, krótko przed wylęgiem, maleje ponownie do poziomu diapauzalnego.

2. Wpływ promieniowania gamma zastosowanego w diapauzie jest uchwytny dopiero w okresie rozwoju podiapauzalnego; dawka 5 kr opóźnia syntezę DNA i obniża wylęg gąsienic o 15%; dawka 20 kr opóźnia syntezę DNA, uszkadza syntezę RNA i całkowicie hamuje wylęg; dawka 200 kr hamuje całkowicie syntezę DNA i powoduje rozpad RNA.

3. Wrażliwość jaj na dawkę 20 kr jest mniejsza, jeśli stosuje się napromieniowanie po okresie, w którym zawartość RNA w jajach osiąga maksimum.

4. Uszkodzenie syntezy RNA w okresie gastrulacji jest prawdopodobnie przyczyną zaburzeń embriogenezy występujących po dawce 20 kr.

Received 15 March, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

MARIA ERECIŃSKA

THE EFFECT OF FAR ULTRAVIOLET LIGHT ON MITOCHONDRIA

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

1. Far UV-light induces rapid and extensive swelling of rat liver mitochondria which depends upon the time of exposure and the amount of protein present in the irradiated mixture. 2. Presence of ATP and Mn^{2+} or Mg^{2+} ions delays the swelling. 3. Formation of lipid peroxide during irradiation appears to be the most likely explanation for the changes resulting in disintegration of the mitochondrial membrane. 4. Ubiquinone disappears from the irradiated mitochondria and the succinate dehydrogenase activity is inhibited to a great extent. The protein of succinate dehydrogenase seems to be the primary target by which the UV-light is absorbed. 5. The protective effect of albumin and nucleotides is unspecific and connected with their high absorption in the UV region.

Sarachek & Townsend (1953) showed that irradiation with ultraviolet light of the yeast cells containing an abundance of large mitochondria resulted in swelling and disruption of these mitochondria. Further investigations revealed that during exposure of the isolated rat liver mitochondria to the UV-light the activity of succinate dehydrogenase and cytochrome oxidase as well as oxidative phosphorylation were partially damaged (Dallam & Anderson, 1957; Beyer, 1959a). Beyer & Vennison (1958) and Beyer (1960) reported that during irradiation occurred a release of various nucleotides and inactivation of dinitrophenol-stimulated ATPase and ATP-P_i exchange reaction. The extensive studies of Beyer (1959b, 1961) showed that the destructive effect of the far UV-light on the activity of succinate dehydrogenase and oxidative phosphorylation could not be reversed by the addition of a number of UV-absorbing compounds including FAD, NAD and coenzyme Q (Beyer, 1959b). However, the presence of ATP and serum albumin during irradiation afforded a considerable degree of protection of succinate-linked oxidative phosphorylation system (Beyer, 1959b). Beyer was unable to explain the mechanism of the inhibitory effect of ultraviolet light; he suggested that it might be absorbed by multiple targets and that disruption of the electron transfer system is too diverse to be reversed by the compounds studied.

The purpose of the present work was to cast some light on the effect of the far UV-light (below 300 mμ) on the physical state of the mitochondria as well as on the primary targets by which the UV-light is absorbed in actively respiring and phosphorylating mitochondria.

MATERIALS AND METHODS

Rat liver mitochondria were isolated in 0.33 M-sucrose - 5 mM-tris - 1 mM-EDTA medium, pH 7.4, and ox heart mitochondria in sucrose-phosphate-EDTA medium as described previously (Szarkowska & Erecińska, 1965; Erecińska & Szarkowska, 1965). Mitochondria were irradiated using medium pressure Hg-lamp (220 v, 3.5 A, 257 mμ max.) at 0°. The distance between the source of ultraviolet light and the surface of the irradiated mitochondria was kept constant at 6 cm. Mitochondria were poured on Petri dish in a thin layer of 1 - 2 mm. and irradiated through the Co-Ni filter. Mitochondria were irradiated in 0.33 M-sucrose - 25 mM-tris - 1 mM-EDTA medium, pH 7.4, or in 0.175 M-KCl - 25 mM-tris - 1 mM-EDTA medium, pH 7.4, for different periods of time and the samples withdrawn to check the swelling and the activity.

Changes of volume (swelling) were followed as a decrease in extinction at 545 mμ in the same medium as used for irradiation in a total volume of 2.9 ml. for at least 20 min. An appropriate amount of mitochondrial protein was used to give the initial extinction between 0.5 and 0.6.

Lipid peroxide formation was followed by the thiobarbituric acid method (Ottolenghi, 1959) as described by Hunter, Gebicki, Hoffsten, Weinstein & Scott (1963). Since sucrose interferes with this reaction, KCl-tris medium was used in these experiments.

Succinate oxidation was determined by: (1), Oxygen uptake measured polarographically with Clark oxygen electrode in the medium used for irradiation; P_i was 2 mM, ADP 0.2 mM and succinate 10 mM in a final volume of 1 ml. (2), Manometrically by the phenazine methosulphate method as described by Keilin & King (1960) in a system composed of 60 mM-phosphate buffer, pH 7.4, 1 mM-KCN, 40 mM-succinate and an appropriate amount of mitochondria in total volume of 3.0 ml. After the temperature equilibration (30°) 2 mg. of phenazine methosulphate were added from the side arm. The activity was determined by the rate of oxygen uptake measured during the first 10 min. (3). By reduction of cytochrome *c* measured by the increase in extinction at 546 mμ in the recording Eppendorf spectrophotometer. The medium contained 60 mM-phosphate buffer, pH 7.4, 1 mM-KCN, 10 mM-succinate, 0.05 mM-cytochrome *c* and appropriate amount of mitochondria. The results were calculated using the millimolar extinction coefficient $\varepsilon mM = 9.8 \times cm.^{-1}$. (4), By the reduction of exogenous ubiquinone measured in a medium consisting of 0.25 M-sucrose, 10 mM-phosphate buffer, pH 7.4, 1 mM-KCN, Triton X100 1.2 mg., 0.290 μmoles of UQ_6 and the mitochondria in a total volume of 1 ml., according to the procedure described by Szarkowska & Drabikowska (1963). Incubation was 3 min. at 37°.

Cytochrome oxidase activity was measured polarographically at 20° in a system containing 50 μmoles of phosphate buffer, pH 7.4, 10 μmoles of ascorbate and 0.07 μmole of cytochrome *c*.

The endogenous ubiquinone was determined as described previously (Erecińska

& Szarkowska, 1965). Protein was determined by the biuret method as described by Szarkowska & Klingenberg (1963).

All the chemicals used were commercial products: ADP, ITP, GTP of Sigma Chemical Co. (St. Louis, Mo., U.S.A.), ATP of Pabst (Milwaukee, Wisc., U.S.A.), albumin of Calbiochem (Los Angeles, Calif., U.S.A.), UQ_6 of Farmochimica Cutalo-Calosi S.p.a. (Napoli, Italy) and phenazine methosulphate of Light and Co. Ltd. (Colnbrook, Bucks, England).

RESULTS

Ultraviolet light-induced swelling of the mitochondria

Rat liver mitochondria prepared in sucrose-tris-EDTA medium were very resistant to any changes in volume; they did not swell even when kept for an hour at room temperature. Figure 1 shows the results of a typical experiment on the far UV-light induced changes of the mitochondrial volume. This rapid and extensive

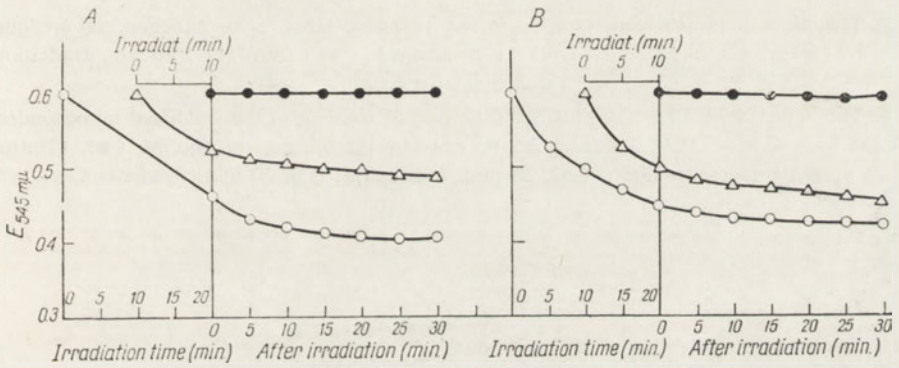


Fig. 1. The effect of ultraviolet light on mitochondrial swelling: *A*, in sucrose-tris-EDTA medium (5.7 mg. mitochondrial protein/ml. of the irradiated mixture), and *B*, in KCl-tris-EDTA medium (5.9 mg. mitochondrial protein/ml.); (●), control; (△), 10 min. irradiation; (○), 20 min. irradiation.

swelling occurred in the course of irradiation and was observed both in sucrose-tris-EDTA and KCl-tris-EDTA medium. The extent of swelling depended upon the time of exposure to the UV-light and was critically influenced by the concentration of mitochondrial protein in the mixture used for irradiation (Fig. 2). Raising the concentration of protein might prevent at least partially the effect of irradiation. If, however, the time of exposure was long enough swelling occurred even in a thick mixture. A number of agents were tested for their ability to inhibit or reverse mitochondrial swelling induced by UV-light; electron transfer chain inhibitors (1 mM-KCN, 1 mM-azide), metal chelating agents (10 mM-citrate, 1 mM-EDTA) and fatty acid binding substance (albumin, 10 mg./ml.) did not reverse the process and when present during irradiation did not afford any protective effect. The curves thus obtained followed those obtained in Fig. 1. Only the addition of 1.5 mM-ATP

together with Mg^{2+} or Mn^{2+} ions afforded a partial protection which could be overcome by increasing the time of irradiation (Fig. 3). Many conditions found in the irradiated mitochondria were optimal for lipid peroxide formation. For these reasons the formation of material giving thiobarbituric acid colour test during

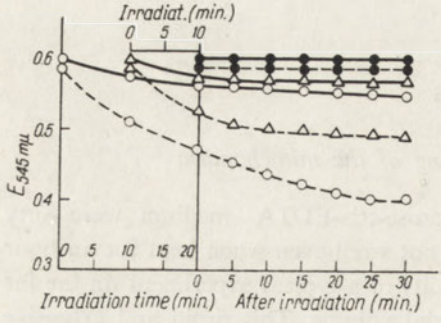


Fig. 2

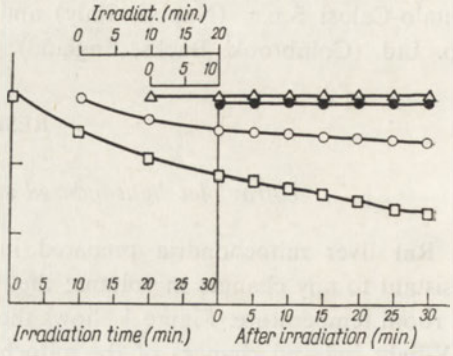


Fig. 3

Fig. 2. The effect of protein concentration in the irradiated mixture on mitochondrial swelling: (——), 8.9 mg. protein/ml.; (— — —), 4.3 mg. protein/ml.; (●), control; (△), 10 min. irradiation; (○), 20 min. irradiation.

Fig. 3. The influence of ATP and $MnCl_2$ on the rate of swelling of the irradiated mitochondria. Medium: sucrose-tris-EDTA, 1.5 mM-ATP, 0.7 mM-$MnCl_2$; 6.1 mg. protein/ml. (●), Control; (△), 10 min. irradiation; (○), 20 min. irradiation; (□), 30 min. irradiation.

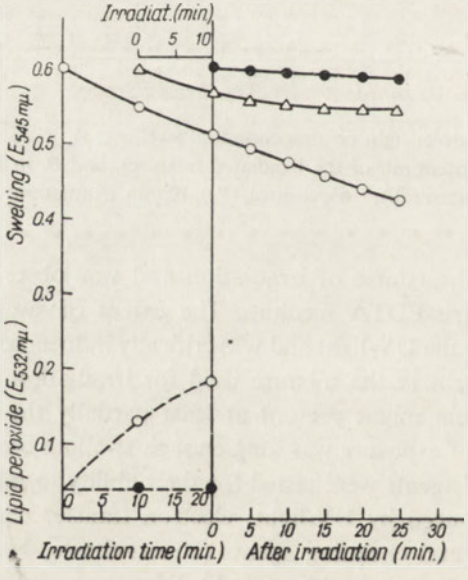


Fig. 4. Swelling of irradiated mitochondria and formation of lipid peroxide (lower part of the diagram) in KCl-tris-EDTA medium containing 5.7 mg. protein/ml. (●), Control; (△), 10 min. irradiation; (○), 20 min. irradiation.

UV-light induced swelling was investigated (Fig. 4). There was no increase in lipid peroxide in the control sample while already in the first 10 min. of irradiation the amount of material giving thiobarbituric acid colour was sufficiently high to explain the recorded swelling. It should be mentioned that under our experimental conditions lipid peroxide formation was followed in the presence of EDTA in the medium.

The influence of ultraviolet light on succinate oxidation

To study the changes taking place in the irradiated mitochondria, different electron acceptors were used to check the activity of succinate oxidizing system. Oxygen was used as the terminal electron acceptor, phenazine methosulphate as the very immediate one and the exogenous ubiquinone and cytochrome *c* as the traps for electrons in the central part of the respiratory chain. It can be seen in Table 1 that already after 10 min. of irradiation the activity of succinate : oxygen electron transport declined to about 50% of the control. The activities of succinate : cytochrome *c*, succinate : ubiquinone electron transport and succinate dehydrogenase measured by phenazine methosulphate method were also impaired. The loss of activity took place in the aerobic as well as in the anaerobic conditions. The activity

Table 1

The effect of ultraviolet light on succinate oxidation in rat liver mitochondria

Conditions as described under Methods. A, Oxygen uptake determined polarographically. B, Succinate dehydrogenase activity measured manometrically by phenazine methosulphate method.

Treatment	Oxygen uptake (μg. atoms/mg. protein/min.)		Cytochrome *c* reduced (μmoles/mg. protein/min.)	UQ_6 reduced	
	A	B		(μmoles)	(%)
None	0.089	0.159	0.176	0.220	74
10 min. irradiation	0.041	0.124	0.114	0.103	35
20 min. irradiation	0.008	0.060	0.026	0	0

Table 2

The effect of ultraviolet light on endogenous ubiquinone and succinate-cytochrome c reductase activity in ox heart mitochondria

Conditions as described under Methods. Ubiquinone was assayed as described previously (Erecińska & Szarkowska, 1965).

Treatment	Total UQ (μmoles/g. protein)	Cytochrome *c* reduced (μmoles/mg. protein/min.)
None	4.26	0.443
10 min. irradiation	2.81	0.193
20 min. irradiation	1.45	0.088

of succinate : oxygen chain declined at the same rate in two simultaneous experiments one of which was performed in the atmosphere of oxygen and the second in the atmosphere of nitrogen (nitrogen was bubbled through the incubation mixture during irradiation; data not shown).

Since ubiquinone absorbs very strongly in the UV region it was interesting to check its behaviour under the influence of the UV-light. Table 2 shows the effect of irradiation on the content of ubiquinone in ox heart mitochondria. In the first 10 min. about 35% of ubiquinone disappeared and the activity of succinate - cytochrome *c* reductase diminished by 55%.

Table 3 presents the data on cytochrome *c* oxidase activity of the irradiated mitochondria. It can be seen that under our experimental conditions the activity was not impaired after 10 min. irradiation and it began to decline slightly after 20 min.

Table 3

The effect of ultraviolet light on cytochrome c *oxidase of rat liver mitochondria*

Conditions as described under Methods.

Treatment	Oxygen uptake (μg. atoms/mg. protein/min.)
None	0.305
10 min. irradiation	0.315
20 min. irradiation	0.226

Table 4

The effect of various compounds on restoration of succinate-cytochrome c *reductase activity after irradiation of rat liver mitochondria*

Additions	Cytochrome *c* reduced (μmoles/mg. protein/min.)	
	control	after 10 min. irradiation
None	0.158	0.078
ATP (1.5 mM)	0.192	0.080
Albumin (10 mg./ml.)	0.148	0.069
ADP (1.5 mM)	0.160	0.070
UQ_6 (100 μg./ml.)	0.160	0.075
FAD (20 μg./ml.)	0.155	0.077
EDTA (5 mM)	0.155	0.076

Attempts were made to restore the activity of the impaired succinate - cytochrome *c* reductase. Table 4 presents the list of compounds tested and it can be seen that none of them when added to the incubation mixture after irradiation was able to restore the activity. Since Beyer (1959b) reported that ATP and albumin afforded a marked protective effect, we exposed mitochondria to the UV-light in their presence.

Table 5

The protectory effect of nucleotides and albumin on succinate-cytochrome c *reductase activity of irradiated rat liver mitochondria*

Conditions as described under Methods. Nucleotides and albumin were present in the incubation mixture during irradiation.

Additions	Cytochrome *c* reduced (μmoles/mg. protein/min.)		
	Control	after 10 min. irradiation	after 20 min. irradiation
None	0.150	0.068	0.021
ATP (1.5 mM)	0.190	0.088	0.088
Albumin (10 mg./ml.)	0.150	0.085	0.090
ATP (1.5 mM)+albumin (10 mg./ml.)	0.175	0.110	0.100
ADP (1.5 mM)	0.155	0.075	0.072
ITP (1.5 mM)	0.145	0.070	0.068
GTP (1.5 mM)	0.147	0.074	0.072

Table 5 shows that indeed both ATP and albumin exerted some protective effect. This effect was better visible after 20 min. of irradiation; during the first 10 min. the activity dropped somewhat and then remained constant while in control samples it declined sharply. ADP, ITP, and GTP had the same effect. Mitochondria irradiated for 10 min. in the presence of albumin did not lose respiratory control (unpublished data) although succinate dehydrogenase activity decreased. ADP, ITP or GTP added instead of albumin were almost ineffective in protecting the respiratory control of the irradiated mitochondria.

DISCUSSION

Data concerning the mitochondrial swelling have been summarized in recent comprehensive reviews by Lehninger (1962) and Chappell & Greville (1963). Our investigations indicate that the UV-light is another powerful agent causing mitochondrial swelling. The changes in volume which occur are very rapid and extensive, and result in a complete lysis of mitochondria. It has been known that exposure of cells, mitochondria and other biological material to UV-light catalyses the formation of lipid peroxide (Barber & Ottolenghi, 1957). It was found that under our experimental conditions formation of lipid peroxide is characteristically associated with UV-induced changes of the mitochondrial volume. A wide variety of chemical substances was shown to inhibit this oxidation. Barber & Ottolenghi (1957) found that cyanide, glutathione and EDTA inhibited lipid peroxide formation in homogenates exposed to UV-light. Under our experimental conditions none of the agents tested such as electron chain inhibitors, metal-chelating agents or fatty acid binding substances was able to protect against UV-light-induced changes. The discrepancy between our data and those of Barber & Ottolenghi (1957) might be due to the fact that we used a very powerful source of UV-light and under such conditions

the protective effect of all the agents tested might have been too small to counteract the changes induced by irradiation. Therefore it may be concluded that formation of lipid peroxide induced by far UV-light is the most likely explanation for the changes which result in permeability increase and later disintegration of the mitochondrial membrane.

In agreement with Beyer (1959b) it was observed that UV-light is absorbed by multiple targets in the mitochondria. One of such targets is undoubtedly ubiquinone. Brodie (1965) reported that irradiation damaged ubiquinone by about 80%, FAD by 43%, and did not affect the cytochromes. Bishop (1961) found that during 1 min. of irradiation plastoquinone content of chloroplasts fell by about 40%. It can be seen from our experiments that mitochondrial ubiquinone is sensitive to irradiation and disappears from the irradiated mitochondria. The addition of this compound fails, however, to restore the activity of succinate-cytochrome *c* reductase; thus the disappearance of ubiquinone is not the only lesion of the respiratory chain. Data presented in Table 1 indicate that the respiratory chain is impaired prior to the point of location of ubiquinone. The activity of succinate dehydrogenase checked with phenazine methosulphate (which is generally considered to be the very immediate acceptor for this enzyme) is already damaged within the first 10 min. of irradiation. Therefore the second target which absorbs the UV-light in the mitochondria appears to be the protein of succinate dehydrogenase. It is interesting that the protein of succinate dehydrogenase is more sensitive to irradiation than the protein of cytochrome oxidase. The latter enzyme is damaged by irradiation too, it requires, however, longer exposure to UV-light. We may therefore suggest that the effect of UV-light on mitochondria and the drop in succinate dehydrogenase activity are connected very closely with general damaging effect of UV-light on proteins (McLaren & Shugar, 1964). Therefore irradiation will damage all the enzymic reactions of the mitochondria and the rate of inactivation will depend upon the individual sensitivity of various proteins to the UV-light. Here we find an explanation for the finding of Beyer & Vennison (1958) and Beyer (1960) that irradiation inactivates dinitrophenol-stimulated ATPase and ATP-P_i exchange reaction. In agreement with this author it was found that ATP and albumin protect mitochondria during irradiation. However, our data indicate that ADP, ITP and GTP exhibit a similar though less pronounced effect. All these compounds absorb very strongly in the UV-region and therefore their effect is not specific, and they simply act by screening the UV-sensitive targets of the mitochondria. The more specific effect of ATP and albumin cannot, however, be excluded. Serum albumin is an efficient coupling agent and thus it may be effective in protecting the oxidative phosphorylation. ATP on the other hand might increase the intracellular level of this compound thus increasing the respiratory tightness of the mitochondria.

The author wishes to express her gratitude to Professor Dr Józef Heller for his encouragement and helpful discussion during the course of this work, and to Mrs. Elżbieta Kuligowska for her help in carrying out some of the experiments.

REFERENCES

Barber A.A. & Ottolenghi A. (1957). *Proc. Soc. Exp. Biol. Med.* **96**, 471.
Beyer R.E. (1959a). *Radiat. Res.* **9**, 92.
Beyer R.E. (1959b). *Arch. Biochem. Biophys.* **79**, 269.
Beyer R.E. (1960). *Exptl. Cell Res.* **21**, 217.
Beyer R.E. (1961). *Biochim. Biophys. Acta* **48**, 389.
Beyer R.E. & Vennison R.D. (1958). *Biochim. Biophys. Acta* **28**, 432.
Bishop N.I. (1961). In *Ciba Foundation Symp. on Quinones in Electron Transport Chain* (G.E.W. Wolstenholme & C.M. O'Connor, eds.) p. 385. J. & A. Churchill Ltd., London.
Brodie A.F. (1965). In *Biochemistry of Quinones* (R.A. Morton, ed.) p. 356. Academic Press, London - New York.
Chappell J.B. & Greville G.D. (1963). *Separation of subcellular structural components. Biochem. Soc. Symp.* **23**, 39.
Dallam R.D. & Anderson W.W. (1957). *Biochim. Biophys. Acta* **25**, 439.
Erecińska M. & Szarkowska L. (1965). *Acta Biochim. Polon.* **12**, 291.
Hunter F.E., Jr., Gebicki L.M., Hoffsten D.E., Weinstein J. & Scott A. (1963). *J. Biol. Chem.* **238**, 828.
Keilin D. & King T.E. (1960). *Proc. Royal Soc.* **152**, 163.
Lehninger A.L. (1962). *Physiol. Rev.* **42**, 467.
McLaren A.D. & Shugar D. (1964). *Photochemistry of proteins and nucleic acids.* Pergamon Press, Oxford.
Ottolenghi A. (1959). *Arch. Biochem. Biophys.* **79**, 355.
Sarachek A. & Townsend G.F. (1953). *Science* **117**, 31.
Szarkowska L. & Drabikowska A.K. (1963). *Life Sciences*, no. 7, 519.
Szarkowska L. & Erecińska M. (1965). *Acta Biochim. Polon.* **12**, 179.
Szarkowska L. & Klingenberg M. (1963). *Biochem. Z.* **338**, 674.

WPŁYW DALEKIEGO ULTRAFIOLETU NA MITOCHONDRIA

Streszczenie

1. Daleki ultrafiolet powoduje pęcznienie mitochondriów, które zależy od czasu naświetlania iilości białka w mieszaninie.

2. Obecność ATP i jonów Mn^{2+} lub Mg^{2+} opóźnia pęcznienie.

3. Wydaje się, że przyczyną pęcznienia mitochondriów w czasie naświetlania jest tworzenie się nadtlenków lipidów.

4. W czasie naświetlania znika z mitochondriów ubichinon i zostaje uszkodzona dehydrogenaza bursztynianowa. Ultrafiolet jest pierwotnie absorbowany przez samo białko enzymu.

5. Ochronny wpływ nukleotydów i albuminy jest niespecyficzny, związany z ich dużą absorpcją w ultrafiolecie.

Received 26 April, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

H. VENNER

TAXONOMY OF *SARCINA* ON THE BASIS OF THEIR DNA BASE COMPOSITION

Institut für Mikrobiologie und experimentelle Therapie, Jena, der Deutschen Akademie der Wissenschaften zu Berlin

Dedicated to Prof. Dr. J. Heller on his 70th anniversary

1. Nucleic acids were isolated by different methods from various strains of *Sarcina*. 2. The isolated nucleic acids were contaminated with polysaccharide, especially those from the *Zymosarcina*, and with polyphosphate (*Urosarcina*). 3. By the isolation procedure the DNA could be concentrated so as to permit reliable determination of the purine and pyrimidine content. 4. The purine and pyrimidine bases showed the same regularities as those observed in other micro-organisms. The guanine+cytosine content, which is known to be species-dependent, varied for the subgenera of *Sarcina*. 5. The taxonomic relationship in connection with some concepts on the evolution of *Sarcina* is discussed on the basis of the guanine+cytosine content of DNA.

In 1950 Chargaff (cf. 1955) studying the base composition of DNA from different sources observed by paper chromatography regularities on which the double-helix model of the DNA was founded by Watson & Crick (1953). All the DNA samples analysed showed the same molar content of adenine and thymine as well as guanine and cytosine, and the sum of purines equalled that of pyrimidines, whereas the sum of G+C was found to range in the limits of nearly 25 to 75 mole%. Ki Yong Lee, Wahl & Barbu (1956) and Beloserski & Spirin (1960) summarized the results on the GC content of the DNA from micro-organisms and pointed to a correlation between the corresponding values and the taxonomic relationship. Later Sueoka (1961) and Marmur, Rownd & Schildkraut (1963) confirmed these results by the T_m technique, a method in which the melting temperature of DNA is measured by UV spectrophotometry. With few exceptions, which mostly could be due to errors in the taxonomical classification, the various species belonging to the same family exhibited a similar GC content. Since the two species of the genus of *Sarcina* (*S. lutea* and *S. flava*) studied by Ki Yong Lee *et al.* (1956) and Beloserski & Spirin (1960) revealed a very high GC content deviating strongly from the other members of the family of Micrococcaceae it was necessary to examine the DNA of other subgenera of *Sarcina*. For this purpose methods for the isolation of DNA from this micro-organism were developed and the base composition of the isolated DNA was measured by paper chromatography.

ORGANISMS

Sarcina lutea. A medium containing in 100 ml. 1 g. of glucose, 2 g. of sodium lactate, 0.9 g. K_2HPO_4, 0.2 g. KH_2PO_4, 0.1 mg. of nicotinamide, 20 g. of vitamin-free hydrolysate of casein and the usual inorganic salts (pH 7.0 - 7.2) was inoculated with *Sarcina lutea* SG 140 in a 0.5 l. flask and cultivated for 48 hr. at 27°. Three ml. of the culture were taken as inoculum for a flask with freshly prepared medium and the bacteria cultivated for 4 days at 27° with vigorous aeration. The bacterial cells from 25 flasks were collected by centrifuging (3100 *g*, 45 min., 4°) and the sediment (61.7 g. wet wt.) resuspended in 0.068 M-NaCl - 0.01 M-sodium citrate solution and centrifuged in the same manner.

Zymosarcina. The medium contained in 100 ml. 3 g. of saccharose, 2 g. of casein peptone, 1 g. of malt extract, and was acidified by H_3PO_4 to pH 4.8. Sterilization was carried out by heating at 100° for 45 min. in an autoclave in the presence of N_2 (the stream of N_2 was passed till cooling to room temp.). The precultivation was done by vigorous shaking in a 500 ml. flask containing 100 ml. of the medium inoculated by either *S. ventriculi*, strain 1 - 3, or *S. maxima*, strain M1 or M2. For the main cultivation the contents of 5 flasks were transferred to 10 l. of freshly prepared medium in a 20 l. flask in the presence of N_2 and incubated for 5 hr. Then a stream of N_2 was passed until the culture produced abundant amounts of CO_2; then the incubation was continued without N_2. After the CO_2 liberation had diminished (30 hr.), the flask was cooled to 4° (final pH 4.4 - 4.5), the supernatant removed by siphoning, the bottom layer (10 cm.) centrifuged and the bacteria resuspended as described for *S. lutea*.

Urosarcina. The medium contained in 100 ml. of meat broth 1 g. of peptone, 0.3 g. NaCl and 0.2 g. $Na_2HPO_4 \cdot 12\ H_2O$; pH 7.2 - 7.4. Three ml. of the medium were inoculated with *S. ureae* SG 943 or *S. agilis* SG 936. After 2 days at room temp. without shaking or stirring, the culture was used for inoculation of 100 ml. of freshly prepared medium in a 500 ml. flask. This was stored again for 2 days under the same conditions, then the content of 3 flasks was used as inoculum per 10 l. of medium in a 20 l. flask, sterilized at 100° for 45 min. Following 4 days incubation (*S. ureae* at 20°, *S. agilis* at 37°) the bacteria were harvested by centrifugation at 4200 *g* for 15 min. and resuspended as described for *S. lutea*.

METHODS

Isolation of DNA. The DNA was isolated by different methods: (1), Incubation of the bacterial suspension with lysozyme and grinding with glass powder (Vendrely, Palmade & Vendrely, 1956). (2), Homogenization with quartz powder and isolation with dodecylsulphate (Kay, Simmons & Dounce, 1952). (3), Incubation with lysozyme in tris buffer (Scaletti & Naylor, 1959) and further treatment with dodecylsulphate (Gandelman, Zamenhof & Chargaff, 1952); the supernatant after dodecylsulphate treatment was added with 3 vol. of ethanol and the precipitate was called fraction *a* (final preparation; cf. Table 1); the sediment after dodecylsulphate treatment

was dissolved in 0.15 M-NaCl and treated with phenol in the usual manner (fraction *b*). In expt. 16 two kinds of sediments were obtained on precipitation with ethanol: a fibrous one (fraction *a*) and a flocky one (fraction *c*; predominantly RNA) which were separated mechanically. (4), Isolation of DNA together with RNA using phenol and *p*-aminosalicylate as described earlier (Venner, 1963a, method 7). Fractions *a* and *b* correspond to the same fractions as mentioned in method (3). (5), Isolation of DNA alone with phenol and *p*-aminosalicylate (Kirby, 1957) as modified by Venner (1963a, method 6).

Analytical methods. Nitrogen, phosphorus and H_2O content were determined as described previously (Venner, 1963a). The DNA samples after acid hydrolysis were tested for sugars by paper chromatography (Venner, 1963b). The DNA and RNA content in the bacterial cells and in the isolated DNA was estimated by the methods of Webb (1955, 1956) in a modification already described (Venner, 1963a). The UV spectra were determined with a Hilger Uvispek spectrophotometer.

Determination of the base composition. Nucleic acid (mixture of DNA and RNA), 80 - 200 mg., was dissolved in 3 - 5 ml. of 0.5 N-KOH and incubated for 18 hr. at 37°. After cooling to 0° the samples were neutralized with 30% $HClO_4$ and by further addition of the acid adjusted to 1 - 3% free acid. The precipitate ($KClO_4$, protein, DNA) was separated by centrifugation and washed with ice-cold 1% (w/v) $HClO_4$ (supernatant and washings contained the ribonucleotides).

For determination of bases of the RNA, the combined supernatant and washings were adjusted, either immediately or after neutralization, to a volume of 25 ml. with 1 N-KOH at 0°, and $KClO_4$ was centrifuged off. Two 10-ml. samples were dried by infrared light at 70° over P_2O_5 nearly to dryness (24 hr.). The residue was hydrolysed with 0.01 ml. of 70% (w/v) $HClO_4$ in a boiling water bath. After dilution with 1 ml. water and short heating, the solution was freed from carbon by centrifugation followed by repeated washing of the sediment with portions of 0.2 ml. H_2O till no base could be detected with the purine lamp in the last washings (drained on paper). The combined supernatant and washings were made up to 5 ml. and determined by paper chromatography as described earlier (Venner, 1960). In some cases the solution was neutralized with NaOH before being adjusted to the exact volume.

For determination of bases of the DNA, the obtained precipitate containing the DNA, protein and $KClO_4$, was mixed with a solution of saturated sodium bicarbonate to pH 8.0 - 8.5, then extracted with 5 ml. of 10% NaCl solution for 1 hr. at 100°. $KClO_4$ and protein were centrifuged off and washed with saline solution. The DNA from the combined supernatant and washings was precipitated by adding 3 vol. of ethanol. After standing for 24 hr. at 4°, the DNA was separated by centrifugation in the cold, washed twice with 70% (v/v) and finally with 96% (v/v) ethanol, dried for 1 hr. at 100° and hydrolysed with 0.1 ml. $HClO_4$ (70%, w/v). Then the mixture was heated with 0.5 ml. H_2O and freed from carbon as described for RNA. The combined supernatant and washings were adjusted to 5 ml. or concentrated to 1 ml. by a stream of warm air passing over the surface in a special cuvette, and the base content was determined as above.

Table 1

Isolation of DNA from different species and strains of Sarcina

The cultivation of the bacteria and the isolation procedures are described in the text. Carbohydrates were tested in the isolated nucleic acid; in expts. 6, 7, 8, 10 and 11, **galactose**, **glucose**, **mannose**, xylose, fucose, **arabinose**, rhamnose, and ribose were found; in expts. 14 and 16b, **ribose**; in expt. 13, **ribose**, xylose, **arabinose** (main constituents are in bold type). No test for deoxyribose was performed.

Expt. no.	No. of preparat. combined	Bacteria (g.) wet	Bacteria (g.) dry	Strain	Isolation method ***	Yield (mg.)	Yield (%)	$E^{1\%}_{1\,cm}$	λ_{max} (mμ)	Content (%)* DNA	Content (%)* RNA	Protein (biuret reaction)
1	1	101.8	16.7	*S. lutea*	1	440	2.4	13.0	257	0.25 (3.11)	3.40 (4.97)	?
2	2	30.9	5.8	*S. lutea*	4*a*	187	3.0	4.1	260	—	—	—
					b	60	0.9	130	265	—	—	?
3	1	72.4	15.4	*S. ventriculi* 1 - 3	1	260	1.5	end absorption		—	—	+
4	3	321.7	54.9	*S. ventriculi* 1 - 3	4	2 069	3.5	43.4	258	0.08 (1.50)	9.4 - 12.2 (4.89)	+
5	1	61.0	14.6	*S. maxima* M1+M2	1	900	5.6	13.0	257	(3.11)	(4.97)	?
6	6	424.5	94.2	*S. maxima* M1+M2	4	5 610	5.5	27.4 - 98.5	260	2.4 (0.75)	23.8 - 26.1 (5.18)	+
7	8	578.2	117.0	*S. maxima* M1	4	5 032	3.9	19.6 - 107.1	258	(0.58)	(7.99)	+
8	7	474.1	91.4	*S. maxima* M2	4	5 711	5.4	46.7 - 85.7	258	(0.58)	(7.80)	+
9	1	73.8	14.6	*S. maxima* M1	2	502	3.1	40.0	257	—	—	+
10	1	12.0**	—	*S. maxima* M2	3*a*	2 380	17.9	end absorption		—	—	+
					b	122	0.9	49.8	257	—	—	?
11	4	212.3	44.0	*S. agilis*	4	1 543	3.1	122.2	258	1.63	38.9	—
12	1	44.0	7.8	*S. agilis*	5	19	0.2	147.8	258	—	—	—
13	1	11.8	2.2	*S. agilis*	3*a*	194	8.0	65.8	258	—	—	+
					b	26	1.1	115.7	259	—	—	—
14	4	329.9	53.9	*S. ureae*	4	1 321	2.2	158.4	258	3.6	29.0 - 45.0 (1.05-4.85)	—
15	3	197.8	33.3	*S. ureae*	5	203	0.5	152.5	257 - 9	—	—	?
16	1	84.0	13.9	*S. ureae*	3*a*	247	1.6	82.6	259	—	—	+
					b	112	0.7	155.2	259	—	—	—
					c	502	3.3	35.9	259	—	—	+

* Values in parentheses: content per dry weight. ** Freeze-dried. *** *a*; *b*; *c* represent different fractions (see text).

The qualitative test for purines and pyrimidines in the bacterial cells was carried out in a similar manner.

For the determination of the bases in the pure DNA the samples were hydrolysed directly with concentrated $HClO_4$.

RESULTS

Isolation of DNA. The results obtained for the five species studied by different isolation procedures are shown in Tables 1 and 2. The method (1) of Vendrely *et al.* (1956) appeared to have low efficiency, as low extinction values were obtained for the DNA isolated from *S. lutea* (expt. 1), *S. ventriculi* (expt. 3) and *S. maxima* (expt. 5). These preparations contained only small amounts of DNA and were mainly composed of polysaccharides. Nearly the same was valid for the method (2) with dodecylsulphate (expt. 9). In general, the isolation with the phenol methods was more efficient and the results were dependent on the subgenus of *Sarcina* used. In method (4) the differences appeared already in the formation of the layers during phenol treatment; with *S. ventriculi* a broad interphase was observed, with *S. maxima* it was narrow, and with *S. lutea* it could not be seen. Consequently, the phenol layer of *S. lutea* was rich in nucleic acids, mainly RNA, in contrast to the *Zymosarcina*. The smaller was the formation of the interphase, the more concentrated was the nucleic acid in the phenol phase. In connection with this, the yield of nucleic acid from the upper water phase became smaller. Also in the case of isolating DNA from all *Sarcina* by method (4) it was observed that a great part of the nucleic acid remained insoluble after each ethanol precipitation. Especially in an experiment with *S. lutea*, the insoluble residue after solubilization of the first ethanol precipitate showed a high DNA content, $E^{1\%}_{1\,cm}$ at 265 mμ being 130 (expt. 2b), whereas the final preparation exhibited only a value of 4 at 260 mμ (expt. 2a). Such a behaviour is characteristic for nucleoprotein. If the spectrophotometric data ($E^{1\%}_{1\,cm}$ at λ_{max}) of the DNA isolated by the method (4) from the different strains are compared, the lower values (less than 100) spread over a great range obtained for *Zymosarcina* are obviously in contrast with the data for all the other *Sarcina*. The difference

Table 2

Analytical data of the DNA isolated from Sarcina *by method* (4)

The methods are described in the text.

Expt. no.*	Strain	H_2O (%)	N (%)	P (%)	N/P (atom ratio)	ε(P) at λ_{max}
4	*S. ventriculi* 1 - 3	8.54	9.93	4.13	5.32	3 255
6	*S. maxima* M1+M2	7.92	11.20	5.0	4.95	6 100
11	*S. agilis*	12.98	9.34	12.23	1.69	3 095
14	*S. ureae*	10.64	11.80	9.44	2.76	5 200

* Cf. Table 1.

Table 3

Base composition of the nucleic acids of Sarcina

The analytical method is described in the text. The DNA of *S. ureae* was additionally purified by dodecylsulphate treatment.

DNA sample*	Strain		Bases found (mole %)					Pu/Py	6-keto/6-amino	G+C (mole %)	Content of DNA (% of weight)
			G	A	C	T	U				
2	*S. lutea*	DNA	35.4	14.1	35.3	15.2	—	0.98	1.02	70.7	0.25
		RNA	30.9	26.1	24.0	—	19.0	1.32	1.00	54.9	3.40
3	*S. ventriculi*	DNA	20.2	29.6	21.6	28.6	—	1.00	0.96	41.8	0.08
		RNA	33.5	22.5	25.0	—	19.0	1.27	1.10	58.5	9.40
		RNA**	9.1	26.2	38.9	—	25.4	0.55	0.53	48.0	—
5	*S. maxima*	DNA	23.4	35.3	16.4	24.9	—	1.42	0.94	39.8	2.40
		RNA	29.4	26.4	24.2	—	20.0	1.26	0.98	53.6	26.10
11	*S. agilis*	DNA	27.0	25.6	24.1	23.3	—	1.11	1.01	51.1	1.63
		RNA	31.5	23.5	28.4	—	16.6	1.22	0.93	59.9	38.90
14	*S. ureae*	DNA	22.3	28.2	22.4	27.1	—	1.02	0.98	44.7	24.90
		RNA	32.9	22.6	29.1	—	17.6	1.15	1.02	62.0	45.0

* Cf. Table 1.
** Neutralized after hydrolysis.

between the two strains of *S. maxima*, M1 and M2, also seems to be interesting. The isolation of DNA by the method (5) (Expt. 12, 15) and the method (3) of Scaletti & Naylor (1959), gave similar results to those obtained by method (4). Only method (5) yielded a fibrous DNA free from RNA.

The DNA of *Zymosarcina* was contaminated with considerable amounts of polysaccharides which are believed to derive from the cell wall (cf. footnote in Table 1). All attempts to purify DNA samples of *Zymosarcina* by various methods (chloroform - amylalcohol according to Sevag, Lackmann & Smolens, 1938; treatment with chymotrypsin or 2-methoxyethanol, Kirby, 1956) failed, although the Kirby method has been developed especially for the separation of polysaccharides. As already stated by Kirby (1956), it is difficult to purify a nucleic acid containing high amounts of impurities and it is more convenient to start a new isolation procedure. The DNA samples of *Urosarcina* were contaminated with polyphosphate, as revealed by high phosphorus content; therefore the values of ε(P) were low in spite of the normal extinction in the UV light (see Table 2).

In conclusion, it should be admitted that the results of the isolation of nucleic acids from *Sarcina*, especially *Zymosarcina*, are not completely satisfying. This could be explained by the fact that the cells of *Zymosarcina* are very large, and the content of nucleic acids, especially DNA, is low. New methods *via* the protoplasts avoiding the interference by components of the cell wall seem to be more promising (unpublished results). However, the nucleic acids isolated in this work allowed to perform reliable analyses of the base composition.

Base composition. Table 3 shows the base composition of the isolated nucleic acids. In RNA bases of *S. ventriculi* (neutralized before paper chromatography), the guanine content was found to be very low (9.1 mole%). This was due to the low solubility of the guanine at neutral pH, its main part remaining on the start of the chromatogram. Also the content of adenine was too low. In all the other data for RNA and DNA of *Sarcina*, presented in Table 3 (which represent the results of chromatography of non-neutralized hydrolysates), the regularities already observed in various micro-organisms (Beloserski & Spirin, 1960) were found. In DNA the ratio purine/pyrimidine, resp. 6-keto/6-amino-group containing bases was nearly 1.0; only the purine/pyrimidine ratio for DNA of *S. maxima* exhibited a greater deviation, and that of *S. agilis* a small one. This deviation was not due to the low DNA content because *S. ventriculi* showed a normal purine/pyrimidine ratio although the DNA content of the sample used for the analytical determination was extremely low. The deviation was due to a brownish pigment which arose after hydrolysis with $HClO_4$ and gave coloured tailing on the chromatogram. When formic acid was used for hydrolysis, the pigmentation effect grew stronger. The pigment seemed to derive from a polysaccharide of the cell wall (chitin?), which was found to be present in the nucleic acid sample.

In the RNA fraction the ratio 6-keto/6-amino groups was also found to be 1.0, whereas the purine/pyrimidine ratio was 1.15 - 1.32. It should be mentioned that, due to the conditions of isolation, the results of the determination of the base

Table 4

Carbohydrates occurring in the nucleic acid fraction of different groups of micro-organisms

The main components are in bold type; minor constituents are set in parentheses. Hexosamines, diaminopimelic acid, muramic acid were not tested, resp. not registered.

Taxon	Carbohydrates	Reference
Sarcina	**Gal Glc Man** Xyl Fuc **Ara** (Rha)	this work
Basidiomycetes	**Gal Glc Man** Xyl Fuc	Venner (1963a)
Mycobacteria	Gal Glc Man **Ara**	Venner (1963b)
Proteus	**Gal Glc**	Sarfert & Venner (1965)

composition concern only the high-molecular fractions of the nucleic acids of the cell. So far the values for the RNA have been referred mainly to the ribosomal RNA.

The determination of the base composition of the DNA could be performed only after concentration of the nucleic acids, because of the very low content of DNA in the whole cell and the presence of a very high amount of the RNA, especially in *Zymosarcina*.

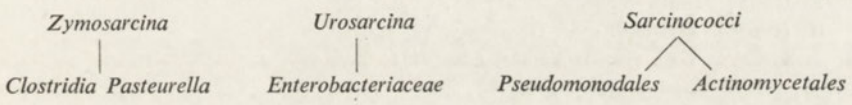
Carbohydrates occurring in the nucleic acid fraction. The results are presented in Table 4; for comparison, the results for other groups of micro-organisms are included. Obviously, there seems to exist a taxonomic specificity for the carbohydrates, which could be based on the genetically determined occurrence of enzymes which participate in the biosynthesis of polysaccharides. Such a correlation is known to exist in the polysaccharides specific for the blood groups (Wiener, 1948).

DISCUSSION

Since the first experiment of Avery, MacLeod & McCarty (1944), the role of DNA as the genetic material carrying the information for the whole cell of micro-organism, has been fully recognized. It is not surprising that attempts were made to establish a correlation between the composition of DNA and the taxonomic classification (Sueoka, 1961; Marmur, Falkow & Mandel, 1963). Up till now it was impossible to elucidate the nucleotide sequence of a DNA molecule. Therefore all these studies on taxonomy refer to the guanine+cytosine content, which represents a parameter measurable by the actually available methods. The results proved to be very useful and stimulating, but there are some important restrictions which can be summarized as follows. 1. It is necessary to ensure that the results of determinations refer only to the informational DNA of the cell, and also that only one kind of DNA molecules is taken for analysis. These conditions can be fulfilled only in the lower organisms, where one kind of DNA is representative for the whole genetic information of the cell. The DNA of higher organisms should be fractionated

before analysis to separate the molecules according to their different nucleotide sequence. 2. An unequivocal taxonomic classification should be based on the nucleotide sequence of the DNA; if the classification is based only on the guanine + cytosine content, this may affect the reliability of the obtained results. Then the interpretation of the taxonomic relationships is limited by the same regularities, as G + C content and nucleotide sequence are connected together.

For a discussion of the results presented in this paper it is indispensable to take the foregoing into consideration. On the basis of the guanine + cytosine content for the genus of *Sarcina*, in agreement with the taxonomic system of Bergey (1957), we can differentiate three main groups (subgenera): *Zymosarcina*, *Urosarcina* and *Sarcinococci*. However, in contrast to other genera or even families, these three groups differ very much from each other. Whereas the *Zymosarcina* belong to the extreme adenine-thymine type, the *Sarcinococci* represent the extreme guanine-cytosine type, and the *Urosarcina* are just in the middle position, representing the type of equality (guanine + cytosine = adenine + thymine). Such great differences were observed up till now only between orders, or occasionally one family of an order or one species of a genus was apart from its taxon. Therefore it appears that there is no evidence for a simple genetic relationship between these three groups of *Sarcina*. This follows also from the regularities between base content and sequence mentioned above in point 2. If we look for a correlation with the other organisms, the following picture results:



Taking into account the morphological similarity and other reasons which led the taxonomists to group together the different *Sarcina* and, on the other hand, the differences in their metabolism (e.g. anaerobiosis or aerobiosis) and in the base composition of DNA, the taxon of *Sarcina* seems to be a group which represents a common morphological level deriving from different bacteria, and is directed toward similar organization of cell formations, rather than a genetically closely related taxon. Further work which is now in progress will contribute to elucidate this problem.

I gratefully acknowledge the excellent technical assistance of Miss Chem.-Ing. Eva Sarfert throughout this work. I am also indebted to Professor Dr. H. Knöll and to Professor Dr. W. Köhler for the cultures of *Zymosarcina*, resp. *Urosarcina* and *Sarcinococci*, and also for their helpful advice.

REFERENCES

Avery, O.T., MacLeod, C.M. & McCarty, M. (1944). *J. exptl. Med.* **79**, 137.
Beloserski, A.N. & Spirin, A.S. (1960). In *The Nucleic Acids* (E. Chargaff & J.N. Davidson, eds.) Vol. III, p. 147. Academic Press, New York and London.
Bergey's Manual of Determinative Bacteriology (1957; reprinted 1959). (R.S. Breed, E.G.D. Murray & N.R. Smith, eds.) The Williams & Wilkins Co., Baltimore.
Chargaff, E. (1950). *Experientia*, Basel **6**, 201.
Chargaff, E. (1955). In *The Nucleic Acids* (E. Chargaff & J.N. Davidson, eds.) Vol. I, p. 307. Academic Press, New York.
Gandelman, B., Zamenhof, S. & Chargaff, E. (1952). *Biochim. biophys. Acta* **9**, 399.
Kay, E., Simmons, N. & Dounce, A. (1952). *J. Amer. chem. Soc.* **74**, 1724.
Kirby, K.S. (1956). *Biochem. J.* **64**, 405.
Kirby, K.S. (1957). *Biochem.* J. **66**, 495.
Ki Young Lee, Wahl, R. & Barbu, E. (1956). *Ann. Inst. Pasteur* **91**, 212.
Marmur, J., Falkow, S. & Mandel, M. (1963). In *Annual Review of Microbiology* (C.E. Clifton, S. Raffel & M.P. Starr, eds.) Vol. 17, p. 329. Annual Reviews Inc., Palo Alto, Calif. U.S.A.
Marmur, J., Rownd, R. & Schildkraut, C.L. (1963). In *Progress in Nucleic Acid Research* (J.N. Davidson & W.E. Cohn, eds.) Vol. 1, p. 232. Academic Press, New York & London.
Sarfert, E. & Venner, H. (1965). *Hoppe-Seyler's Z. physiol. Chem.* **340**, 157.
Scaletti, J. & Naylor, H. (1959). *J. Bact.* **78**, 422.
Sevag, M.G., Lackmann, D.B. & Smolens, S. (1938). *J. biol. Chem.* **124**, 425.
Sueoka, N. (1961). *J. mol. Biol.* **3**, 31.
Vendrely, R., Palmade, Ch. & Vendrely, C. (1956). *Nature, Lond.*, **178**, 1044.
Venner, H. (1960). *Hoppe-Seyler's Z. physiol. Chem.* **322**, 122.
Venner, H. (1963a). *Hoppe-Seyler's Z. physiol. Chem.* **333**, 5.
Venner, H. (1963b). *Act. biol. med. Germanica* **11**, 806.
Watson, J. & Crick, F.C. (1953). *Nature, Lond.* **171**, 737; 964.
Webb, J.M. (1955). *J. biol. Chem.* **213**, 107.
Webb, J.M. (1956). *J. biol. Chem.* **221**, 635.
Wiener, A. (1948). *Lancet* **254**, 343.

SYSTEMATYKA *SARCINA* OPARTA NA SKŁADZIE ZASAD KWASU DEZOKSYRYBONUKLEINOWEGO

Streszczenie

1. Kwasy nukleinowe izolowano różnymi metodami z rozmaitych szczepów rodzaju *Sarcina*.

2. Wyizolowane frakcje były zanieczyszczone wielocukrami, szczególnie u *Zymosarcina*, oraz polifosforanem (*Urosarcina*).

3. DNA w czasie izolowania zagęszczano w celu otrzymania pewniejszych wyników oznaczania zasad purynowych i pirymidynowych.

4. Zasady purynowe i pirymidynowe wykazywały te same regularności, jakie obserwowano u innych mikroorganizmów. Zawartość guaniny i cytozyny, która, jak wiadomo, zależy od gatunku, zmieniała się w podrodzajach *Sarcina*.

5. Na podstawie zawartości guaniny i cytozyny w DNA przedyskutowano systematykę rodzaju *Sarcina* w związku z pewnymi teoriami na temat jego ewolucji.

Received 2 May, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

LIDIA D. WASILEWSKA and I. REIFER

URACIL AND URIDINE AS PRECURSORS OF PYRIMIDINE NUCLEOTIDES IN HIGHER PLANTS

Institute of Biochemistry and Biophysics, Polish Academy of Sciences and the Department of Biochemistry, Central College of Agriculture, Warszawa

1. It was shown that [2-^{14}C]uracil and [2-^{14}C]uridine are actively metabolized in wheat, green peas and spinach plants and that they are precursors of pyrimidine nucleotide synthesis. 2. The highest specific activities were detected in 5′-UMP, followed in declining order by 5′-CMP, 2′(3′)-UMP and 2′(3′)-CMP. 3. Uracil may be converted into 5′-UMP by ribosidation followed by phosphorylation, or by direct ribotidation. 4. Uridine is several times more active as precursor of the pyrimidine nucleotides in the investigated plants than is uracil. 5. Plant homogenates retain high uridine kinase activity and some uridine phosphorylase activity.

The work of Wright, Kornberg, Lieberman, Reichard and their collaborators (see review by Reichard, 1959) has established the main path of pyrimidine nucleotide biosynthesis in animal tissues and micro-organisms, known as the orotic acid path:

$$\text{Asp} \xrightarrow{CO_2+NH_3} \text{CAsp} \xrightarrow{-H_2O} \text{DHOA} \xrightarrow{-H_2} \text{OA} \xrightarrow{\text{PRPP}} \text{OMP} \xrightarrow{-CO_2} \text{UMP}$$

Work on pyrimidine nucleotide biosynthesis in higher plants was first undertaken in our laboratory and it was established by Reifer, Buchowicz & Toczko (1960) and Buchowicz, Reifer & Makowski (1961) that young wheat plants fed with CAsp[1] and orotic acid (Buchowicz & Reifer, 1961, 1962) respectively, synthesized UMP with uracil and uridine as possible intermediates.

It has therefore been decided to investigate systematically the anabolic pathway of uracil and uridine in higher plants, particularly as preliminary experiments with [2-^{14}C]uracil on excised wheat plants have shown distinct incorporation of the carbon label from uracil into pyrimidine mono- and polynucleotides (Buchowicz, Wasilewska, Witecki & Reifer, 1963). An additional incentive in the undertaking

[1] Following abbreviations were used throughout this paper: Asp, L-aspartic acid; CAsp, L-carbamoylaspartic acid; CP, carbamoylphosphate; DHOA, L-4,5-dihydro-orotic acid; OA, orotic acid; OMP, orotidine-5′-monophosphate; 5′-UMP, 5′-UDP, 5′-UTP, uridine-5′-mono-, -di, -triphosphate; 5′-CMP, cytidine-5′-monophosphate; 2′(3′)-UMP, uridine-2′(3′)-monophosphate; 2′(3′)-CMP, cytidine-2′(3′)-monophosphate; R-1-P, D-ribose-1-phosphate; R-5-P, D-ribose-5-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate.

of work on those lines in plant material, have been numerous reports suggesting that in animal tissues and micro-organisms, reversion of some stages of reductive degradation of pyrimidines may occur with a possible utilization of the free bases and their decomposition products as precursors of pyrimidine nucleotide biosynthesis (see review by Schulman, 1961).

MATERIAL AND METHODS

Reagents. [2-^{14}C]Uracil and [2-^{14}C]uridine were purchased from the Radiochemical Centre, Amersham, England; uracil, uridine, 5′-UMP, 5′-CMP, 2′(3′)-UMP, 2′(3′)-CMP and 3-phosphoglyceric acid, Ba-salt, from Nutritional Biochemical Corp., Ohio, U.S.A.; D-ribose-5-phosphate, Ba-salt, from A.G. Fluka, Buchs, Switzerland; 5-phosphoribosyl-1-pyrophosphate from L. Light and Corp. Ltd., Colnbrook, England; ATP from T. Schuchardt, Munich, Germany; K_2HPO_4 and KH_2PO_4 from E. Merck, Darmstadt, Germany. The Li-salt of carbamoylphosphate was prepared according to Jones, Spector & Lipmann (1954). ATP-regenerating system was prepared according to Ratner (1955). In one of the first experiments [2-^{14}C]uridine has been obtained biosynthetically using CAsp with ^{14}C in the carbamoyl group (Table 1). All other reagents were of Polish production, distributed by Biuro Obrotu Odczynnikami, Gliwice.

Plants. Winter wheat (*Triticum vulgare*), variety Dańkowska 40, green pea (*Pisum sativum*) variety Perła Szlachetna and spinach (*Spinacia oleracea*) variety Gaudry. Six-day-old wheat plants, 14-day-old pea plants and 16-day-old spinach plants were cut off about 1 cm. above the roots and 2 g. of material used for each experiment. Homogenates were prepared from 10 g. samples.

Feeding and preparation of homogenates. Excised plants were immersed with the cut off ends in small vessels containing usually 1 ml. of the solution with the labelled precursor. Feeding was carried out at 30° at the required length of time and transpiration was accelerated by the use of a small fan. The plant material was then thoroughly washed with water and quickly frozen in solid CO_2. The uptake of substrates was determined from the difference between the amount originally employed and that recovered after feeding and washing of the plant material. When respiratory CO_2 was to be determined, the feeding was conducted under an aerated glassbell and the carbon dioxide collected in a saturated solution of barium hydroxide.

For experiments *in vitro* plant homogenates were prepared at 4° by maceration with the following solution: 10 mM-phosphate buffer, pH 6.5, containing in 1 ml.: 0.2 m-mole saccharose, 0.2 μmole ATP, 0.5 μmole $MgCl_2$, 1.0 μmole 3-phosphoglycerate and 0.12 mg. of ATP-regenerating system. One ml. of the above solution was used per each gram of plant material. The pulp was filtered through a fourfold layer of cheesecloth and 4 ml. of the filtrate was incubated for 1 hr. with the calculated amount of the ^{14}C-labelled substrate at 20°. Samples with zero time of incubation served as controls and as reference of quantitative recovery of the carbon label. All determinations were carried out in duplicates.

Isolation and identification. (a) Acid-soluble fraction. The frozen plant samples were brought into a small mortar that was previously placed in an ice-bath, and ground finely into a uniform powder. The acid-soluble pyrimidine derivatives were extracted several times with about 10 ml. portions of cold 0.3 N-$HClO_4$, made up to a final volume of 50 ml. and centrifuged. The pyrimidines from the supernatant were then adsorbed on activated charcoal using about 100 mg. per 1 g. of plant material. The filtrate after adsorption on charcoal usually still retained small amounts of the compounds to be investigated, showing maximum absorption at 260 mμ. For this reason adsorption was repeated twice with 75 and 50 mg. of charcoal, which was then exhaustively washed with ice-cold water in order to remove the last traces of $HClO_4$. Desorption of the pyrimidines was achieved by washing the charcoal at least 5 times with 30 ml. portions of a boiling mixture of acetone and 0.1 N-ammonia (4 : 1, v/v). The extract was then evaporated to dryness, taken up in 5 ml. of water and adsorbed in a column on Dowex 1×1, Cl^-, 50 - 100 mesh, 1×15 cm.

From the column, free bases and nucleosides were eluted with about 200 ml. of water, 5′-CMP with 200 ml. of 0.003 N-HCl, 5′-AMP with 300 ml. of 0.005 N-HCl and 5′-UMP with 200 ml. of 0.015 N-HCl. The washings were in every case tested spectrophotometrically for quantitative elution. The fractions so obtained were evaporated and partitioned by means of paper chromatography. Pure compounds were obtained on rechromatography. The free bases and the nucleosides were partitioned by descending chromatography in *n*-butanol saturated with water (Buchanan, 1951) and then rechromatographed as described by Wyatt (1955). The 5′-CMP and 5′-UMP fractions were chromatographed according to Wyatt and rechromatographed according to Smith & Markham (1950). The spots were localized on the paper by means of UV photography, cut out, eluted with 0.01 N-HCl and the resulting solution was then submitted to spectrophotometric determinations and radioactivity measurements. Extinction was determined by the use of a Soviet spectrophotometer, type SF-4 and radioactivity measured with a window counter (1.4 mg./cm.^{2}).

Identification of the compounds was based on their R_F values as compared with the corresponding standards, on absorption spectra at pH 2, wavelength from 210 to 320 mμ and determination of minimum and maximum absorption as well as on calculations of the proportions of extinction at $\lambda = 250/260$, 280/260 and 290/260. To determine the range of losses of the isolated compounds, known amounts of standards were added to plant material. On routine procedures it was found that recoveries amounted to 80 - 87%, with average losses of $16.2\%\pm3.7\%$. All results reported are not corrected.

(b) *Acid-insoluble fraction.* After removal of the acid-soluble fraction, the residue containing the polynucleotides was washed several times with cold 0.5 mM non-radioactive uracil or uridine (depending on the ^{14}C-precursor used) dissolved in 0.3 N-$HClO_4$. The non-radioactive substance was then quantitatively removed by washing with ice-cold 0.3 N-$HClO_4$ and the residue hydrolysed to free the 2′(3′)-nucleotides. With few exceptions (Table 1, Table 3, expt. I, and Table 4, expt. VI)

where the agent of hydrolysis was 1 N-$HClO_4$ for 1 hr. at 100° (Loring, 1955), in all other cases alkaline hydrolysis with 0.3 N-KOH for 16 hr. at 37° (Schmidt & Thannhauser, 1945) was employed throughout. The alkaline extracts were acidified with 3 N-$HClO_4$ to a final concentration of 0.3 N of the acid. The precipitate was filtered off, washed several times with 10 ml. portions of cold $HClO_4$ to a final volume of 100 ml. and the residue discarded. The combined supernatant and washings containing the hydrolysed RNA were then partitioned and the products isolated as described above for the 5′-nucleotide fraction. Alkaline hydrolysis was considered preferable, because it does not affect DNA and the ribonucleotides were not contaminated with their deoxy-counterparts. Furthermore RNA on alkaline hydrolysis is split to 2′(3′)-nucleotides only, whereas on acid hydrolysis the nucleotides decompose partly to nucleosides and the free bases, leading to appreciable losses in the quantitative determinations of 2′(3′)-nucleotides.

RESULTS

Seasonal differences and the diurnal rhythm of the plants cause considerable variations in quantitative chemical composition. For this reason the results in subsequent tables were ordered in separate groups (indicated by horizontal delineation) in which the material used originated from the same crop and was harvested at the same time. Results shown in Table 1 indicate that both [2-^{14}C]-uracil and [2-^{14}C]uridine underwent considerable anabolic changes in excised wheat plants and that the changes were detectable already after an incubation period of 30 min. Among the products of uracil anabolism the highest specific activity was detected in uridine, followed by 5′-UMP, 5′-CMP, with the smallest activity in the pyrimidine nucleotides of the 2′(3′) series. With [2-^{14}C]uridine as precursor the highest activity was found in 5′-UMP, followed by 5′-CMP, 2′(3′)-UMP and 2′(3′)-CMP. In both experiments extension of time of feeding resulted in proportional increases of the carbon label in the isolated pyrimidine derivatives.

Uridine appears to be a better precursor of the nucleotides than uracil, as with a 40-fold lower specific activity of uridine the label detected in 5′-UMP amounted to as much as 50% of the activity obtained with the very radioactive uracil (for example: after 4 hr. of feeding).

Comparing the specific activities of 5′-UMP and 5′-CMP further differences between both precursors can be observed. With uracil as precursor, the activity detected in 5′-CMP was about half of the activity detected in 5′-UMP, whereas with uridine as precursor the activity of 5′-CMP was about 8 times lower than that of 5′-UMP. Results reported for the control samples show that within the first 4 hr. of feeding no detectable changes in the quantities of the pyrimidine nucleotides could be observed. Only after 8 hr. of feeding a certain decrease of 2′(3′)-UMP was detected, suggesting a possible degradation of RNA. Therefore in further experiments the time of feeding did not exceed 4 hr. Adenine and guanine were also isolated, but no radioactivity was detected.

As ATP, R-1-P and PRPP participate in the reactions of ribosidation and

ribotidation, it was decided to study the influence of phosphate compounds on the anabolism of uracil and uridine. It has been already reported (Buchowicz *et al.*, 1963) that orthophosphate, ADP and ATP stimulated the incorporation of the ^{14}C-label from uracil into uridine. As can be seen from Table 2, KH_2PO_4, CP and R-5-P stimulated the incorporation of the precursors into all pyrimidine derivatives, whereas PRPP and pyrophosphate caused a decline of the specific activity in these compounds, when compared with the control samples fed with the radioactive precursors only. Comparing the quantities of endogenous pyrimidine derivatives in expt. I with expt. II, it may be seen that the latter ones contained several times less uridine and nearly twice as much 2′(3′)-nucleotides as the first ones. In all experiments reported in this paper, where the endogenous level of uridine amounted to about 0.3 - 0.5 μmole/g. of fresh material, the incorporation of the uracil label into uridine was considerably higher than into 5′-UMP. On the other hand, in the expt. II of Table 2, with the extremely low uridine level of 0.07 μmole/g., the specific activity of 5′-UMP was twice as high as the activity of uridine. Furthermore identical concentrations and specific activities of the uracil used caused in expt. II a six times higher incorporation of the label into 5′-UMP than in expt. I. It is possible that the observed differences resulted from the diurnal rhythm of the plants, as the material with the low uridine content was harvested and fed late in the evening, whereas normally all experiments were carried out at about 2 p.m.

Results reported in Table 3 refer to the influence of various quantities of uracil upon [2-^{14}C]uridine metabolism. It has been assumed that an increased content of cellular uracil, considered to be the first degradation product of uridine, may lower the rate of breakdown of this nucleotide and thus shift the equilibrium in the direction of anabolic changes. Non-radioactive uracil was used in concentrations of 0.2, 4.0 and 40.0 mM, respectively. However, the results obtained were contrary to our expectations and in all cases where non-radioactive uracil has been used, an increase of radioactivity in the uracil was observed. Total radioactivity in the sample fed with non-radioactive 40 mM-uracil (expt. I) was about 15 times higher than in the control plants, where no uracil has been introduced. Even the specific activities of the isolated uracil were higher than in the control, where 0.2 and 4 mM-uracil were added to the feeding mixture, despite the fact that endogenous uracil has been diluted with the non-radioactive compound.

These observations led to the conclusion that increased quantities of uracil present in plant material may cause increased degradation of uridine *via* uracil. Indeed, measurements of radioactivity in the respiratory CO_2 have proved that the increase of ^{14}C in carbon dioxide went hand in hand with the increased total radioactivity found in uracil (expts. II, III).

Results shown in Table 4 additionally indicate that the uridine level has a considerable effect upon 5′-UMP synthesis from [2-^{14}C]uracil. With low amounts of uridine (Table 4, expt. II) the incorporation of uracil into 5′-UMP was several times higher than when uridine was present in the usually detected quantities. When non-radioactive uracil was added to [^{14}C]uridine an increase of the specific activity of 5′-UMP has been observed (expts. IV and VI) and correspondingly the introduction

Table 1

Influence of time of feeding on the amounts and specific activities of pyrimidine derivatives isolated from excised wheat plants fed with [2-^{14}C]*uracil and* [2-^{14}C]*uridine, respectively*

Spec. act. of uracil: 30 000 counts/sec./μmole; of uridine: 740 counts/sec./μmole. The acid-insoluble fraction was hydrolysed with 1 N-$HClO_4$ for 1 hr. at 100°. In this and subsequent Tables, the material used within a separate experiment originated from the same crop and was harvested at the same time; the amounts of pyrimidine derivatives are expressed as μmoles per gram of fresh tissue.

Expt. no.	Feeding		Acid-soluble fraction								Acid-insoluble fraction			
			Uracil		Uridine		5′-UMP		5′-CMP		2′(3′)-UMP		2′(3′)-CMP	
	substrate (1 μmole/ml.)	time (hr.)	μmole/g.	counts/ sec./ μmole	μmole/g.	counts/ sec./ μmole	μmole/g.	counts/ sec./ μmole	μmole/g.	counts/ sec./ μmole	μmole/g.	counts/ sec./ μmole	μmole/g.	counts/ sec./ μmole
I	None	0	0.098	—	0.218	—	0.078	—	0.068	—	0.260	—	0.288	—
	[2-^{14}C]Uracil	0.5	0.110	3002	0.210	101	0.079	9.1	0.070	4.0	0.248	0.6	0.290	0.3
	[2-^{14}C]Uracil	1	0.131	6886	0.202	218	0.073	17.6	0.066	7.5	0.256	1.1	0.290	0.7
	[2-^{14}C]Uracil	2	0.159	9121	0.186	392	0.064	51.4	0.061	22.2	0.240	2.8	0.276	1.6
	[2-^{14}C]Uracil	4	0.212	14967	0.178	688	0.068	90.8	0.064	43.8	0.242	6.3	0.279	4.0
II	None	0	—	—	0.196	—	0.081	—	0.071	—	0.218	—	0.228	—
	[2-^{14}C]Uridine	0.5	—	—	0.212	59	0.076	4.8	0.068	0.5	0.217	0.3	0.226	0.0
	[2-^{14}C]Uridine	2	—	—	0.241	136	0.083	28.4	0.072	3.6	0.221	1.4	0.221	0.0
	[2-^{14}C]Uridine	4	—	—	0.349	282	0.091	41.5	0.065	6.1	0.203	2.3	0.230	0.4
	[2-^{14}C]Uridine	8	—	—	0.552	487	0.088	72.6	0.068	9.6	0.161	4.9	0.220	1.1

Table 2

Amounts and specific activities of pyrimidine derivatives isolated from plants fed with [2-^{14}C]*uracil and* [2-^{14}C]*uridine, respectively, in presence of phosphates*

Spec. act. of uracil: 24 000 counts/sec./μmole; of uridine: 37 000 counts/sec./μmole. Time of feeding: 3 hr.

Plant	Expt. no.	Feeding mixture		Intake (counts/sec./g.)	Acid-soluble fraction								Acid-insoluble fraction			
					Uracil		Uridine		5′-UMP		5′-CMP		2′(3′)-UMP		2′(3′)-CMP	
		labelled substrate (mM)	addition (mM)		μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole
Pea	I	[2-^{14}C]Uracil, 0.5	None	4562	0.253	3108	0.342	326	0.135	51	0.112	26	0.886	2.8	0.907	1.3
		[2-^{14}C]Uracil, 0.5	KH_2PO_4, 10	4538	0.246	3012	0.321	414	0.141	66	0.121	27	0.902	3.2	0.902	1.6
		[2-^{14}C]Uracil, 0.5	R-5-P, 10	4114	0.238	2942	0.350	388	0.144	65	0.116	28	0.902	3.0	0.944	1.6
Pea	II	[2-^{14}C]Uracil, 0.5*	CP, 2	2100	0.168	4602	0.072	73	0.163	183	0.109	12	1.641	4.0	1.483	0.6
		[2-^{14}C]Uracil, 0.5*	PRPP, 2	2100	0.173	5636	0.070	53	0.142	131	0.103	7	1.596	2.9	1.418	0.4
		[2-^{14}C]Uracil, 0.5**	KH_2PO_4, 10	3870	0.192	5918	0.066	168	0.122	369	0.103	18	1.615	7.3	1.388	2.1
Pea	III	[2-^{14}C]Uracil, 0.5	CP, 10	4562	0.244	2940	0.313	416	0.151	73	0.118	28	0.986	3.3	1.012	1.8
		[2-^{14}C]Uracil, 0.5	$K_4P_2O_7$, 10	4380	0.238	3558	0.308	269	0.146	41	0.126	20	1.004	1.7	0.953	1.0
Pea	IV	[2-^{14}C]Uridine, 0.33	None	5020	0.222	288	0.398	3741	0.148	481	0.122	161	1.056	28.0	1.083	8.4
		[2-^{11}C]Uridine, 0.33	KH_2PO_4, 10	5236	0.231	242	0.386	3904	0.141	619	0.118	190	1.007	30.0	1.061	9.1
		[2-^{14}C]Uridine, 0.33	CP, 10	5502	0.235	244	0.400	3956	0.144	606	0.124	183	1.036	29.1	1.092	9.4
Wheat	V	[2-^{14}C]Uridine, 0.33	None	5092	0.091	2161	0.242	4915	0.084	316	0.064	48	0.592	21.1	0.494	6.4
		[2-^{14}C] Uridine, 0.33	KH_2PO_4, 10	5173	0.084	2012	0.239	5304	0.078	379	0.068	62	0.604	21.8	0.523	6.2
		[2-^{14}C]Uridine, 0.33	CP, 10	5300	0.084	1984	0.242	5166	0.075	367	0.066	71	0.574	23.6	0.511	7.0

* 1 hr. feeding; incubation mixture 0.5 ml.

** 2 hr. feeding.

Table 3

Amounts and specific activities of pyrimidine derivatives isolated from plants fed with [2-^{14}C]*uridine in presence of non-radioactive uracil*

The feeding mixture contained 10 μmoles of KH_2PO_4/ml. Spec. act. of uridine: 37 000 counts/sec./μmole. Polynucleotides were hydrolysed in expt. I with $HClO_4$, in expts. II and III with KOH. Time of feeding: in expt. I, 4 hr., in expts. II and III, 3 hr.

Plant	Expt. no.	Feeding mixture		Intake (counts/ sec./g.)	$^{14}CO_2$ (counts/ sec./g.)	Acid-soluble fraction									Acid-insoluble fraction			
						Uracil			Uridine		5′-UMP		5′-CMP		2′(3′)-UMP		2′(3′)-CMP	
		[2-^{14}C]-uridine (mM)	uracil (mM)			μmole/g.	counts/ sec./g.	counts/sec./ μmole	μmole/g.	counts/sec./ μmole	μmole/g.	counts/sec./ μmole	μmole/g.	counts/sec./ μmole	μmole/g.	counts/sec./ μmole	μmole/g.	counts/sec./ μmole
Wheat	I	0.33	0.0	3385	—	0.067	30	453	0.270	2391	0.091	865	0.066	146	0.218	27.1	0.205	6.9
		0.33	0.2	3512	—	0.097	66	683	0.222	1927	0.089	1209	0.061	163	0.206	26.6	0.217	7.6
		0.33	4.0	3185	—	0.331	293	887	0.195	1789	0.084	1414	0.060	193	0.199	26.2	0.231	7.1
		0.33	40.0	2920	—	4.256	457	107	0.185	1648	0.069	1413	0.064	202	0.228	28.0	0.220	7.9
Wheat	II	0.25	0.0	1895	33	0.104	32	312	0.236	3144	0.079	391	0.071	77	0.595	37.6	0.524	5.4
		0.25	4.0	1776	68	0.471	275	583	0.207	3064	0.074	458	0.068	98	0.605	39.1	0.501	5.9
Pea	III	0.40	0.0	3410	76	0.233	40	176	0.453	3615	0.144	608	0.118	239	1.005	34.4	0.956	6.3
		0.40	0.2	3425	111	0.275	69	251	0.431	3365	0.138	660	0.120	228	0.986	33.6	0.994	6.6
		0.40	4.0	3162	161	0.573	230	402	0.417	3302	0.141	697	0.125	234	1.035	33.9	0.982	7.2

Table 4

Correlation between uracil and uridine on incorporation of the ^{14}C-label into pyrimidine nucleotides

Specific activity of uracil: 24 000 counts/sec./μmole, of uridine: 37 000 counts/sec./μmole. The feeding mixture contained KH_2PO_4 in 10 mM concn. Polynucleotides in expts. I - V were hydrolysed with KOH, in expt. VI with $HClO_4$. Time of feeding: in expts. I, III, IV and V, 3 hr., in expts. II and VI, 2 hr.

Plant	Expt. no.	Feeding mixture		Intake (counts/sec./g.)	$^{14}CO_2$ (counts/sec./g.)	Acid-soluble fraction									Acid-insoluble fraction			
						Uracil			Uridine		5′-UMP		5′-CMP		2′(3′)-UMP		2′(3′)-CMP	
		labelled substrate (mM)	non-radioactive substrate (μmoles/ml.)			μmole/g.	counts/sec./g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole
Pea	I	[2-^{14}C]Uracil, 0.5	None	4618	—	0.245	686	2795	0.319	402	0.144	70	0.112	29	0.916	3.1	0.974	1.6
Pea	II	[2-^{14}C]Uracil, 0.5	None	3735	—	0.186	1096	5895	0.068	180	0.124	384	0.097	21	1.602	8.4	1.337	1.9
Pea	III	[2-^{14}C]Uracil, 0.5	None	2433	455	0.289	805	2785	0.426	338	0.132	76	0.138	46	0.916	4.0	0.920	2.0
		[2-^{14}C]Uracil, 0.5	Uridine, 2	2380	854	0.400	1044	2610	0.498	194	0.145	50	0.138	37	0.955	2.5	0.964	1.4
Pea	IV	[2-^{14}C]Uridine, 0.4	None	3215	—	0.227	41	184	0.442	3501	0.138	589	0.118	218	1.056	35.1	0.973	8.6
		[2-^{14}C]Uridine, 0.4	Uracil, 0.2	3190	—	0.258	75	289	0.442	3340	0.136	651	0.115	226	1.007	35.9	0.936	7.6
		[2-^{14}C]Uridine, 0.4	Uracil, 4	3055	—	0.550	244	443	0.429	3289	0.132	692	0.122	222	1.124	36.4	0.950	9.4
Pea	V	[2-^{14}C]Uridine, 0.5	None	2560	71	0.245	35	142	0.477	2368	0.149	441	0.133	108	0.991	17.1	0.905	4.7
Wheat	VI	[2-^{14}C]Uridine, 0.2	None	1745	—	0.097	28	292	0.163	1576	0.083	431	0.065	55	0.310	28.2	0.276	6.3
		[2-^{14}C]Uridine, 0.2	Uracil, 0.2	1720	—	0.110	92	835	0.146	1504	0.080	619	0.061	80	0.329	28.9	0.267	6.6
		[2-^{14}C]Uridine, 0.2	Uracil, 40	1467	—	3.287	414	126	0.130	1406	0.072	692	0.059	111	0.317	32.0	0.292	7.4

of non-radioactive uridine together with [^{14}C]uracil decreased the specific activity of 5′-UMP (expt. III).

To ascertain whether the increased catabolism of uridine in presence of uracil is of general nature in plants, the same experiments were carried out on spinach plants *in vivo* and in spinach homogenates. As can be seen from Table 5 the results obtained *in vivo* on spinach plants are comparable to the results reported for wheat and green peas. However, in homogenates the addition of non-labelled uracil did not cause increased catabolism of uridine, and a 25-fold dilution with uracil resulted in a 23-fold drop in the specific activity of uracil upon isolation from the homogenate. It would appear that intact cell structure is necessary for the deribosidation of uridine caused by the addition of uracil.

Table 5

Influence of uracil on [2-^{14}C]*uridine degradation in spinach*

Excised spinach plants were fed for 2 hr. in a mixture of 0.06 mM-[2-^{14}C]uridine with or without the addition of uracil. The homogenates were incubated for 1 hr. in a mixture of 0.06 mM-[2-^{14}C]-uridine, ATP and ATP-regenerating system, with or without the addition of uracil. Spec. act. of uridine: 84 000 counts/sec./μmole.

Material	Uracil (mM)	Intake (counts/ sec./g.)	Uracil			Uridine		
			μmole/g.	counts/ sec./g.	counts/ sec./ μmole	μmole/g.	counts/ sec/g.	counts/ sec./ μmole
Excised plant	0	1920	0.078	84	1077	0.090	476	5288
Excised plant	4	1790	1.034	626	605	0.096	489	5094
Homogenate	0	2520	0.070	256	3657	0.113	1634	14460
Homogenate	4	2520	1.782	290	163	0.108	1540	14259

As the incorporation of the carbon label from uridine into 5′-UMP has been found in all experiments *in vivo*, the same investigations were repeated on plant homogenates. Preliminary experiments on wheat plants with [2-^{14}C]uridine of very high specific activity (84 000 counts/sec./μmole) were barely promising, as the detected activity in the mononucleotide fraction amounted to only about 15 counts/sec./μmole and in the polynucleotide fraction no radioactivity could be detected at all. However, on addition to the incubation mixture of ATP-regenerating system and 3-phosphoglycerate, appreciable incorporation of the uridine label into 5′-UMP and 5′-CMP could be observed in homogenates prepared from wheat, green peas and spinach plants (Table 6). Small but measurable activity was also detected in 2′(3′)-UMP and considerable enzymic degradation of uridine into uracil has also been observed. In the control non-incubated samples some radioactivity was detected in uracil only. The radioactive non-metabolized uridine was recovered to the extent of 80 - 86%, corresponding to the losses previously reported for the experiments *in vivo* (see Methods).

Table 6

Amounts and specific activities of pyrimidine derivatives isolated from plant homogenates incubated with [2-^{14}C]*uridine*

The homogenates were incubated with [2^{14}-C]uridine, ATP and ATP-regenerating system. In experiments with pea and wheat the concentration of uridine was 0.016 mM, spec. act. 370 000 counts/sec./μmole; in experiments with spinach, uridine concn. was 0.030 mM, spec. act. 84 000 counts/sec./μmole. The control samples were deproteinized at zero-time incubation.

Homogenate from	Incubation (hr.)	Acid-soluble fraction										Acid-insoluble fraction			
		Uracil			Uridine			5′-UMP		5′-CMP		2′(3′)-UMP		2′(3′)-CMP	
		μmole/g.	counts/sec./g.	counts/sec./μmole	μmole/g.	counts/sec./g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole
Pea	0	0.226	182	805	0.504	5007	9954	0.118	0	0.131	0	0.961	0	0.912	0
	1	0.241	744	3087	0.510	2559	5017	0.126	842	0.128	274	0.948	1.72	0.856	0.64
Wheat	0	0.081	180	2222	0.252	4678	18563	0.056	0	0.050	0	0.498	0	0.381	0
	1	0.077	272	3554	0.242	3248	13423	0.054	270	0.053	264	0.516	0.95	0.412	0
Spinach	0	0.081	41	506	0.105	2115	24000	0.048	0	0.042	0	0.221	0	0.250	0
	1	0.084	281	3326	0.098	1626	16872	0.047	177	0.040	74	0.216	1.19	0.243	0

DISCUSSION

It has been shown that uracil and uridine are extensively metabolized in plant tissue and that these compounds participate very appreciably in the synthesis of pyrimidine nucleotides. The metabolism of uracil to 5′-UMP may proceed in the following two ways: (a), by consecutive addition of ribose and phosphate catalysed by two separate enzymes, namely uridine phosphorylase (EC 2.4.2.3) and uridine kinase (EC 2.7.1.48) according to the scheme:

1) uracil + R-1-P → uridine + orthophosphate

2) uridine + ATP → UMP + ADP

and (b), by one-step ribotidation of the free base in presence of uracil phosphoribosyltransferase (EC 2.4.2.9):

uracil + PRPP → UMP + pyrophosphate.

Both reactions have been reported to take place in animal tissues and micro-organisms (see review by Schulman, 1961). However, none of the mentioned enzymes have yet been isolated from plant material although Tunis & Chargaff (1960) have obtained from carrot roots a versatile enzyme system which catalysed phosphorylation of purine and pyrimidine ribo- and deoxyribonucleosides, and some nucleotides. This enzyme called by the authors nucleoside phosphotransferase is not specific for uridine.

The synthesis of UMP described in this paper may in fact be the result of uracil metabolism proceeding on both paths, with one or the other dominating, according to the conditions prevailling in the plant tissue. It is assumed that the endogenous level of uridine and polynucleotides in the investigated plant material may determine the preponderance of one path over the other.

5′-UMP that has been synthesized from uracil has undergone further metabolism as witnessed by the detection of considerable radioactivity in the polynucleotide fraction. It is assumed that the incorporation of 5′-UMP into RNA must proceed by way of its phosphorylation into the di- and triphosphate derivatives. These are the substrates for polynucleotide phosphorylase and RNA polymerase, catalysing the incorporation of 5′-nucleotides into the polynucleotide chain.

It would appear that the incorporation of uracil into RNA reported in this paper proceeds *via* uridine polyphosphates as the addition of pyrophosphate caused a drop in the utilization of [2-^{14}C]uracil. It is believed that the increased concentration of pyrophosphate in the tissue caused a shift in the equilibrium thus partly inhibiting the anabolic changes of uracil. The same effect has been observed in presence of PRPP which on degradation increased the pyrophosphate pool. These observations support the results obtained by Kirkland & Turner (1959 a, b), reporting that the incorporation of 5′-UMP into RNA in plants proceeds *via* UDP and UTP.

In all experiments reported in this paper it has been shown that when plants were fed with uracil, the radioactivity in 5′-CMP was of a very high order and amounted in most cases to as much as 50% of the specific activity detected in

5′-UMP. The polynucleotides could not have been the only source of 5′-CMP as their specific activities were several times lower than the activity of the mononucleotide. Although amination of the end products of pyrimidine nucleotide synthesis cannot be excluded, there is some evidence that UTP was not the only acceptor of the amine group.

Evans & Axelrod (1961) in similar experiments on germinating rape seeds have shown that uracil underwent rapid degradation (about 40% of the introduced label was found in respiratory CO_2) and that only small amounts underwent anabolic changes. They assumed that uracil metabolism into UMP proceeds *via* uridine as an intermediate. King, Wang & Waygood (1965) feeding excised wheat plants found the conversion of [2-^{14}C]uracil into UMP only with uridine as an intermediate. The specific activities shown by these authors are extremely low, amounting to about 4 counts/sec./μmole of uridine and 0.03 counts/sec./μmole of UMP. However, their proportions indicate that uracil was converted into UMP *via* uridine similarly as in most cases reported in this paper. In our work the specific activities of uridine as well as of 5′-UMP were about 100 times higher, therefore we were able to detect marked radioactivity in 5′-CMP as well as in the polynucleotide pyrimidines.

Uridine was a better precursor of pyrimidine nucleotides than was uracil as it not only constituted the immediate direct link of 5′-UMP but its breakdown measured by CO_2 formation proceeded at a much lower rate when compared with uracil (Table 4, expts. III and IV). This is in agreement with the observations of Canellakis (1957) who has shown that there is a distinct relation between the ability of the tissue to degrade uracil and the intensity of its utilization in the synthesis of RNA.

The high specific activity of 5′-UMP, constituting from 1/2 to 1/5 of the specific activity of the recovered uridine indicates that plant material contains uridine kinase of very high activity. Experiments *in vitro* with [2-^{14}C]uridine have additionally shown that this nucleoside underwent rapid conversion into 5′-UMP in plant material (Table 6). The specific activity of 5′-UMP amounting to 5 - 15% of the activity of the recovered uridine proves that raw homogenates have retained much of the enzyme catalysing the phosphorylation of this nucleoside. [^{14}C]Uridine was also utilized in the synthesis of 5′-CMP, and the rate of conversion of uridine into both nucleotides, as shown by the addition of ATP-regenerating system, depended upon the constant flow of ATP, which was the phosphate donor for nucleotide synthesis.

The high specific activity of 5′-CMP which in homogenates from wheat plants was equal to that of 5′-UMP, together with the observation that practically no label was detected in 2′(3′)-CMP, strongly supports the suggestion derived from our experiments *in vivo*, namely that amination of uracil derivatives is possible in the early stages of anabolism, prior to the synthesis of UTP. It is possible that in plant homogenates uridine has been primarily aminated, but direct amination of uracil into cytosine when plants were fed with [^{14}C]uracil cannot be excluded. Evans & Axelrod (1961) have shown that the specific activities of uracil and cytosine were of the same order in plants fed with uracil, and Buchowicz & Reifer (1964)

have isolated from young plants fed with [^{14}C]uracil, uridine and cytidine of identical specific activities. The enzymic degradation of uridine into uracil in plant homogenates proves that active uridine phosphorylase was present and, as far as we know, this is the first reference to this enzyme in plant material with destroyed cell structure.

We have demonstrated that orthophosphates stimulated the conversion of uracil and of uridine into 5′-UMP (Table 2). The fact that uridine phosphorylase activity depends upon the concentration of phosphates in the medium (Cannellakis, 1957) may explain the stimulation of uracil conversion into uridine, when the feeding medium has been supplemented with phosphate ions. The increased rate of uridine conversion into 5′-UMP after addition of phosphate could be explained on the assumption that in excised plants phosphate may be the limiting factor in uridine phosphorylation.

Finally, we wish to discuss our observations related to the interaction between uracil and uridine on their respective anabolisms, when both were fed simultaneously to plant material. Results reported in Table 3 show that an increase of uracil concentration in plant material fed with [2-^{14}C]uridine invariably led to higher specific activities of 5′-UMP and, unexpectedly enough, to a rapid increase of radioactivity in the uracil, rising with the increase of added non-radioactive uracil. This last observation strongly suggests that increased concentration of uracil in the plant cell accelerates uridine deribosidation and its degradation into β-alanine, CO_2 and ammonia as witnessed by the nearly doubled radioactivity detected in the respiratory CO_2. The stimulating effect of uracil on the degradation of uridine appears to be connected in some way with the undamaged cell structure, as this effect has not been observed in plant homogenates.

The increased specific activity of 5′-UMP from labelled uridine in plants additionally fed with non-radioactive uracil is probably not due to stimulation of uridine phosphorylation. We assume that the rise of specific activity of 5′-UMP is caused by the direct one-step ribotidation of uracil. Although the enzyme catalysing this reaction i.e. uracil phosphoribosyltransferase has thus far not been reported in plant material, yet there are valid indications suggesting that this reaction may also take place in higher plants. Ross (1965) has recently shown that in cocklebur and bean leaves, uracil as well as uridine are equally good precursors of UMP, which can be explained on the basis that uracil undergoes direct ribotidation into UMP. The two-step reaction must be excluded, as Ross has shown that uracil was not metabolized into uridine. These results are not in full agreement with our observations as in most cases we have found that in plants fed with [^{14}C]uracil, the specific activity of uridine has been the highest and then followed by 5′-UMP, which would suggest the two-step conversion *via* uridine. However, in some of our experiments, when the plant material contained little endogenous uridine, the specific activity of 5′-UMP was twice as high as the activity of uridine, thus indirectly corroborating the results of Ross and suggesting direct ribotidation of uracil, which in this case dominated over the two-step reaction.

Tentatively it would appear that the level of endogenous uridine in the plant determines the dominance of one type of conversion over the other. With uridine

content less than 0.1 μmole/g. direct ribotidation can easily be observed, whereas with more than 0.3 μmole/g. of uridine the reaction of ribotidation is masked by the dominating two-step conversion *via* uridine. To prove this contention, plant material was fed alternatively with labelled uracil and non-radioactive uridine, and non-radioactive uracil and labelled uridine, respectively. The addition of non-radioactive uridine has reduced the incorporation of the uracil label into 5′-UMP by 30%, which was partly due to the dilution of radioactive uridine and partly due to the suppression of direct incorporation of uracil into 5′-UMP caused by the excess of uridine. These findings are in agreement with the results reported by Ross (1965) who observed that the addition of non-radioactive uridine lowered the incorporation of [^{14}C]uracil by more than 80%, despite that his data indicated direct ribotidation to be the only path of uracil metabolism. In addition we have found that non-radioactive uracil caused increased 5′-UMP synthesis, as under identical conditions the specific activity of 5′-UMP was about 50% higher than in the control where no uracil has been added. We therefore conclude that uridine may partly inhibit direct ribotidation of uracil into 5′-UMP.

REFERENCES

Buchanan J.G. (1951). *Nature* **168**, 1091.

Buchowicz J. (1963). *Acta Biochim. Polon.* **10**, 301.

Buchowicz J. & Reifer I. (1961). *Acta Biochim. Polon.* **8**, 25.

Buchowicz J. & Reifer I. (1962). *Acta Biochim. Polon.* **9**, 63.

Buchowicz J., Reifer I. & Makowski J. (1961). *Acta Biochim. Polon.* **8**, 377.

Buchowicz J., Wasilewska L.D., Witecki J. & Reifer I. (1963). *Acta Biochim. Polon.* **10**, 67.

Buchowicz J. & Reifer I. (1964). *Bull. Acad. Pol. Sci. ser. biol.* **12**, 63.

Canellakis E.S. (1957). *J. Biol. Chem.* **227**, 329, 701.

Evans W.R. & Axelrod B. (1961). *Plant Physiol.* **36**, 9.

Jones M.E., Spector L. & Lipmann F. (1954). *J. Amer. Chem. Soc.* **76**, 5571.

King J., Wang D. & Waygood E.R. (1965). *Canad. J. Biochem.* **43**, 237.

Kirkland R.J.A. & Turner J.F. (1959a). *Biochem. J.* **72**, 716.

Kirkland R.J.A. & Turner J.F. (1959b). *Biochim. Biophys. Acta* **32**, 422.

Loring M.C. (1955). In *The Nucleic Acids* (E. Chargaff & J.N. Davidson, eds.) vol. 1, p. 191. Acad. Press, New York.

Ratner S. (1955). In *Methods in Enzymology* (S.P. Colowick & N.O. Kaplan, eds.) vol. 2, p. 358. Acad. Press, New York.

Reichard P. (1959). *Advanc. Enzymol.* **21**, 263.

Reifer I., Buchowicz J. & Toczko K. (1960). *Acta Biochim. Polon.* **7**, 29.

Ross C. (1965). *Plant Physiol.* **40**, 65.

Schmidt G. & Thannhauser S.J. (1945). *J. Biol. Chem.* **161**, 83.

Schulman M.P. (1961). In *Metabolic Pathways* (D.M. Greenberg, ed.) vol. 2, p. 389. Acad. Press, New York and London.

Smith J.D. & Markham R. (1950). *Biochem. J.* **46**, 509.

Tunis M. & Chargaff E. (1960). *Biochim. Biophys. Acta* **37**, 257, 267.

Wyatt G.R. (1955). In *Nucleic Acids* (E. Chargaff & J.N. Davidson, eds.) vol. 1, p. 243. Acad. Press, New York.

URACYL I URYDYNA JAKO PREKURSORY NUKLEOTYDÓW PIRYMIDYNOWYCH W ROŚLINACH WYŻSZYCH

Streszczenie

1. [2-^{14}C]Uracyl i [2-^{14}C]urydyna są czynnymi metabolitami w pszenicy, grochu i szpinaku oraz są prekursorami nukleotydów pirymidynowych.

2. Najwyższą specyficzną aktywność wykazano w 5′-UMP, następnie w 5′-CMP, 2′(3′)-UMP i 2′(3′)-CMP.

3. Uracyl może być przekształcany do 5′-UMP dwiema drogami poprzez przyłączenie rybozy i fosforanu, lub poprzez bezpośrednią rybotydację.

4. Urydyna jest kilkakrotnie wydajniejszym prekursorem nukleotydów pirymidynowych niż uracyl.

5. Homogenaty roślinne wykazują wysoką aktywność kinazy urydynowej i niższą fosforylazy urydynowej.

Received 27 April, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

LIDIA D. WASILEWSKA and I. REIFER

COMPARISON OF ANABOLISM OF ^{14}C-LABELLED OROTIC ACID, URACIL AND URIDINE IN EXCISED PEA PLANTS

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

1. Excised pea plants were fed with [6-^{14}C]orotic acid, [2-^{14}C]uridine or [2-^{14}C]uracil to compare the degree of utilization of each precursor in the synthesis of pyrimidine nucleotides. 2. Orotate was found to undergo rapid metabolism into uridine, 5′-UMP and uracil. 3. In plants, the formation of 5′-UMP may proceed *via* OMP or alternatively by decarboxylation of orotic acid and orotidine to uracil and uridine, respectively.

Excised plants fed with [2-^{14}C]uracil or [2-^{14}C]uridine may utilize these substances in the synthesis of pyrimidine nucleotides (Buchowicz, Wasilewska, Witecki & Reifer, 1963; Wasilewska & Reifer, 1967). It was also shown (Buchowicz & Reifer, 1962) that [6-^{14}C]orotate is incorporated in wheat mainly into three compounds: uridine, 5′-UMP and uracil, in declining order. This suggested that in plant material orotate may be metabolized into UMP also *via* uracil and uridine as intermediates. In this paper we report about the role of these derivatives in the metabolism of orotate in pea plants.

MATERIAL AND METHODS

Reagents. [6-^{14}C]Orotic acid was obtained from the Radiochemical Centre, Amersham, England; all other reagents were purchased from sources as reported previously (Wasilewska & Reifer, 1967).

Plants and feeding. Excised 10-day-old pea plants, variety Perła Szlachetna were fed for 3 hr. with [6-^{14}C]orotate, [2-^{14}C]uridine or [2-^{14}C]uracil. In some of the experiments radioactive orotate or uracil were supplemented with non-labelled uridine. The feeding procedure was as described previously (Wasilewska & Reifer, 1967).

To compare quantitatively the degree of utilization of the labelled precursors, they were diluted with the corresponding non-radioactive substance so that the specific activities of each amounted to 24 000 counts/sec./μmole. The volume of the feeding mixture was 1 ml., containing 0.5 μmole of the radioactive substrate and 10 μmoles of KH_2PO_4. When required, 2 μmoles of uridine was added.

Methods. The analytical procedure of isolation and identification as well as determinations of specific activities of the analysed pyrimidine derivatives were as described in the preceding paper (Wasilewska & Reifer, 1967). Radioactive

orotate was recovered from the plants upon addition of 0.5 μmole/g. fresh material of orotic acid used as carrier. After elution of 5′-UMP from the Dowex column, orotic acid was quantitatively eluted by washing with additional 300 ml. of 0.015 N-HCl, then isolated by ascending paper chromatography, as described by Leone & Scale (1950).

RESULTS

From the results presented in Table 1 (expts. 1, 3 and 4) it may be seen that the rates of utilization of the precursors are distinctly different. The results of incorporation of [2-^{14}C]uridine and [2-^{14}C]uracil confirm our previous observations (Wasilewska & Reifer, 1967) and offer additional information concerning their metabolism in comparison with the anabolic changes of [6-^{14}C]orotate.

Orotate was rapidly metabolized in pea plants to uridine, 5′-UMP and uracil, similarly as it has already been shown in the wheat (Buchowicz & Reifer, 1962). The highest specific activity was detected in uridine, with slightly lower values for 5′-UMP and comparatively high specific activity in uracil, amounting to over 60% of the isolated uridine. The negligible label found in the recovered orotate proved additionally that this compound underwent rapid metabolism.

The results of expts. 2 and 5 confirmed our previous observations that uridine is an intermediate of uracil metabolism to 5′-UMP (Buchowicz *et al.*, 1963; Wasilewska & Reifer, 1967) and that uridine shows the highest specific activity of all the investigated compounds when the plant is fed with [6-^{14}C]orotate (Buchowicz & Reifer, 1962). It was expected that the addition of non-radioactive uridine, which is an intermediate in the case of both precursors of 5′-UMP, will decrease the specific activity of this nucleotide in proportion to the part taken by uridine in the reaction. In agreement with our expectations, a 10% and 30% dilution effect on 5′-UMP was observed when orotate or uracil were used as precursors (see expts. 2 and 1; 5 and 4).

The specific activities of the isolated uridine were nearly identical, irrespective whether uridine itself or orotate was the radioactive precursor used. Yet the specific activities of 5′-UMP were distinctly different: with orotate as precursor the activity of this nucleotide was 90%, with uridine as precursor only 20% of the specific activity of the isolated uridine. This was not the case with 5′-CMP and the polynucleotides; the specific activities were practically the same after feeding with orotate despite the fact that the activity of 5′-UMP from orotate as precursor was five times higher than from uridine. Similarly, the specific activities of 2′(3′)-UMP bore no relation to that of 5′-UMP, as after feeding with uracil and uridine the label detected in the polynucleotide amounted to 5%, whereas after feeding with orotate to only 1% of the respective specific activity of 5′-UMP.

The total sum of radioactivity recovered in all the pyrimidine derivatives amounted to 15% after feeding with orotate, 5% for uridine and 1% for uracil, calculated on the intake equal to 100%, thus indicating the degree of utilization of the particular precursor.

In similar experiments on 20-day-old plants, identical results have been obtained.

Table 1

Isolation of pyrimidine derivatives from pea plants fed with radioactive orotic acid, uridine and uracil

Specific activity of the radioactive precursors was 24 000 counts/sec./μmole. Time of feeding 3 hr. The amounts of pyrimidine derivatives are expressed as μmoles per gram of fresh tissue.

Expt. no.	Feeding mixture		Intake (counts/sec./g.)	Acid-soluble fraction										Acid-insoluble fraction			
				Uracil		Uridine		5′-UMP		5′-CMP		Orotate		2′(3′)-UMP		2′(3′)-CMP	
	labelled substrate (mM)	addition (mM)		μmoles/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./g.*	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole
1	[6-^{14}C]Orotate, 0.5	KH_2PO_4, 10	2705	0.242	1651	0.455	2704	0.154	2421	0.141	132	0.448	254	1.281	24.9	1.202	6.5
2	[6-^{14}C]Orotate, 0.5	KH_2PO_4, 10 +uridine, 2	2725	0.358	1730	0.512	2055	0.154	2193	0.141	109	0.442	180	1.252	18.7	1.148	6.1
3	[2-^{14}C]Uridine, 0.5	KH_2PO_4, 10	2635	0.251	126	0.493	2668	0.151	468	0.144	113	—	—	1.231	23.7	1.190	5.5
4	[2-^{14}C]Uracil, 0.5	KH_2PO_4, 10	2476	0.302	2312	0.433	369	0.128	91	0.134	48	—	—	1.174	4.1	1.128	1.7
5	[2-^{14}C]Uracil, 0.5	KH_2PO_4, 10 +uridine, 2	2460	0.404	2224	0.505	209	0.142	61	0.137	40	—	—	1.205	2.9	1.131	1.2

* Orotate was isolated after adding 0.5 μmole of this compound (used as carrier) per 1 g. fresh weight.

DISCUSSION

The results reported in this paper confirm previous observations on the metabolism of orotate (Buchowicz & Reifer, 1962) as well as of uracil and uridine (Wasilewska & Reifer, 1967) and supply additional information concerning the emergence of uracil and uridine in the metabolism of orotate in higher plants. Thus it has been confirmed that the products of orotate metabolism are primarily uridine, 5′-UMP and uracil and that uracil and uridine can act as precursors of pyrimidine nucleotides in plant material. Additional observations were possible because specific activities of the pyrimidine derivatives were measured on material obtained and analysed under identical conditions with all three labelled precursors and compared with the results obtained with radioactive orotate or uracil in presence of non-radioactive uridine.

The sum of radioactivity recovered in the mono- and polynucleotides proved that orotate is undoubtedly the most effective and uracil the least effective precursor. The specific activity of 5′-UMP from plants fed with [^{14}C]orotate was 25 times higher than that of 5′-UMP synthesised from [^{14}C]uracil and 5 times higher than when [^{14}C]uridine was used as precursor. This shows conclusively that neither uracil nor uridine are intermediates on the main path of orotate metabolism, despite the fact that orotate may be their parent substance from which they are synthesized as witnessed by their extremely high specific activities. In conjuction with the observation that non-radioactive uridine decreased the specific activities of 5′-UMP in plants fed with [^{14}C]orotate or [^{14}C]uracil, it must be concluded that uridine is an intermediate product in the transformations of orotate into 5′-UMP outside the main orotic acid path. [^{14}C]Uracil had a 50% higher specific activity in plants fed with the radioactive precursor alone than in plants in which the labelled precursor has been diluted with non-radioactive uridine. Corresponding results from samples fed with [^{14}C]orotate were only 10% higher. This would indicate that uridine is the main intermediate of uracil anabolism and is of relatively minor importance in the transformation of orotate into 5′-UMP.

Finally, the specific activities of the uridine isolated from samples fed with [^{14}C]uridine or with [^{14}C]orotate were of the same order, yet the activities of their corresponding 5′-UMP amounted to 20% of uridine activity in the first case and to nearly 90% in the second. The reported data indicate that although uracil and uridine are normal metabolites of orotate in plant material, yet they do not participate as intermediates in the main path of UMP synthesis from orotate.

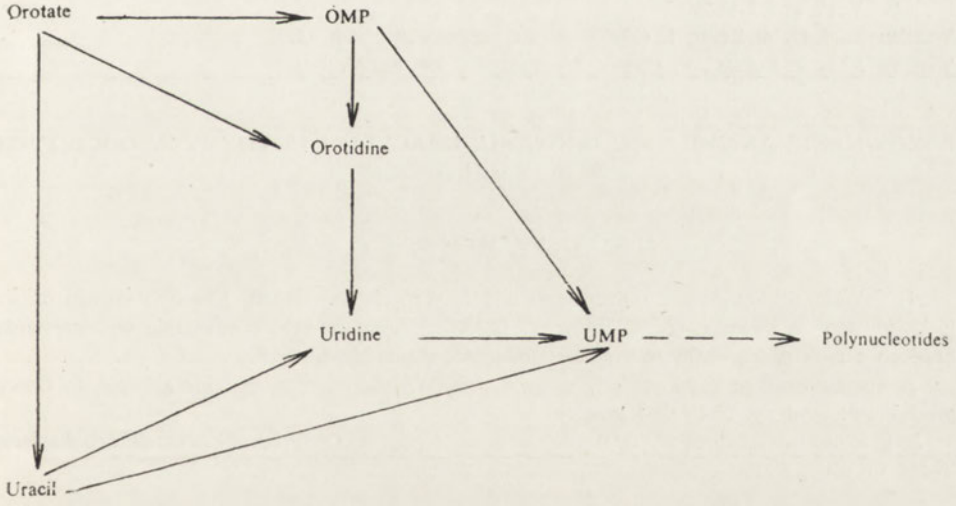
It would appear that in plants the basic path of UMP synthesis from orotate proceeds *via* OMP, identically as described several years ago for micro-organisms and animal tissues (see review by Schulman, 1961). Previous attempts to isolate OMP in our laboratory were unsuccessful (Buchowicz & Reifer, 1962). Recently Kapoor & Waygood (1965) have shown an active spot on radioautograph when the homogenate of wheat embryos was incubated with [^{14}C]orotate. Ross (1965) detected some radioactivity in OMP, but only on addition of 6-azauridine which inhibits its decarboxylation. Although no data were presented in either of the two papers concerning the amounts of OMP found, yet the confirmation of its presence

in plant material together with the detection of plant orotate phosphoribosyltransferase (EC 2.4.2.10) (Kapoor & Waygood, 1965; Wolcott & Ross, 1965) appear to support the view that ribotidation to OMP, followed by decarboxylation to UMP constitutes the main path of orotate anabolism in higher plants.

On the other hand our results indicate that orotate in plant material is also extensively metabolized into uridine, with specific activity identical to that obtained with [^{14}C]uridine as precursor. It is therefore quite obvious that uridine with equal or even higher specific activity than 5′-UMP in samples fed with [^{14}C]orotate cannot to any large extent be a degradation product of the mononucleotide. If this were so the specific activity of uridine would have to be much lower than that of 5′-UMP. From the figures cited it would appear that about 20% of 5′-UMP synthesis in samples fed with orotate originates in anabolic changes *via* uridine, thus supplementing to a marked degree the main orotic acid path. We postulate tentatively that radioactive uridine in samples fed with [^{14}C]orotate may be a product of orotidine decarboxylation, a nucleoside that may originate from OMP and from orotate as well.

The detection of considerable radioactivity in uracil isolated from plants fed with [6-^{14}C]orotate may suggest decarboxylation of orotate rather than its emergence from degradation of 5′-UMP or uridine. This view is supported by very low specific activities of uracil, when uridine was the radioactive precursor. The specific activity of uracil amounts barely to 5% in plants fed with [^{14}C]uridine and to over 60% in plants fed with [^{14}C]orotate, calculated on the basis of the isolated uridine equal to 100%.

The reported data lead to the conclusion that in plants there is an actively functioning anabolic transformation of uracil and uridine, arising from decarboxylation of orotate and orotidine and independent of the main orotic acid path.



Scheme 1. Proposed metabolic paths of orotate, uridine and uracil in higher plants (OMP and orotidine have not been isolated in this work).

Finally it must be noted that [^{14}C]orotate is a more effective precursor than [^{14}C]uridine only in the case of 5′-UMP synthesis, whereas 5′-CMP and the nucleotides of the 2′(3′)-series are equally well synthesized from both precursors. Ross (1965) has also found that in bean leaves, orotate and uridine are utilized to the same extent in the synthesis of RNA and that uracil shows about half of the total activity of the above precursors. His results, however, cannot be fully compared with ours as the content and specific activities of the particular derivatives were not reported. On the basis of the results presented, we suggest that the anabolic transformation of orotate into UMP in plant material may proceed *via* three independent paths: orotate to 5′-UMP *via* OMP as intermediate, similarly as in microorganisms and animal tissues; decarboxylation of orotidine to uridine with subsequent phosphorylation to 5′-UMP and decarboxylation of orotate to uracil and its transformation to 5′-UMP, *via* two alternative paths (Scheme 1) as reported previously (Wasilewska & Reifer, 1967).

We tentatively suggest that the orotic acid path is the main source of 5′-UMP in plant material and that uracil and uridine which arise from orotate metabolism play a very effective part in the synthesis of 5′-CMP and the polynucleotide pyrimidines as well. The transformations of uracil and uridine supplement in independent reactions the classical orotic acid path.

REFERENCES

Buchowicz J. & Reifer I. (1962). *Acta Biochim. Polon.* **9**, 63.
Buchowicz J., Wasilewska L.D., Witecki J. & Reifer I. (1963). *Acta Biochim. Polon.* **10**, 67.
Kapoor M. & Waygood E.R. (1965). *Canad. J. Biochem.* **43**, 143.
Leone E. & Scale E. (1950). *Boll. Soc. Ital. Biol. Sper.* **26**, 1223.
Ross C. (1965). *Plant Physiol.* **40**, 65.
Schulman M.P. (1961). In *Metabolic Pathways* (D.M. Greenberg, ed.) vol. 2, p. 389. Acad. Press, New York and London.
Wasilewska L.D. & Reifer I. (1967). *Acta Biochim. Polon.* **14**, 41.
Wolcott J.M. & Ross C.W. (1965). *Plant Physiol.* **40**, Suppl. lxii

PORÓWNANIE ANABOLIZMU OROTANU, URACYLU I URYDYNY W ODCIĘTYCH ROŚLINACH GROCHU

Streszczenie

1. Odcięte rośliny grochu dokarmiano w identycznych warunkach i w tym samym czasie [6-^{14}C]orotowym kwasem, [2-^{14}C]urydyną i [2-^{14}C]uracylem, w celu porównania wykorzystania każdego z tych prekursorów w syntezie nukleotydów pirymidynowych.

2. Stwierdzono, że kwas orotowy ulega w roślinie intensywnej przemianie głównie do trzech produktów: urydyny, 5′-UMP i uracylu.

3. U roślin powstawanie 5′-UMP może przebiegać poprzez OMP lub też przez dekarboksylację kwasu orotowego albo orotydyny odpowiednio do uracylu i urydyny.

Received 6 May, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

LIDIA D. WASILEWSKA and I. REIFER

SYNTHESIS OF PYRIMIDINE NUCLEOTIDES FROM URACIL AND URIDINE IN STARVED PLANTS

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

1. The rates of transformations of [2-^{14}C]uracil and [2-^{14}C]uridine into pyrimidine nucleotides in starved and non-starved plants were compared. 2. Starvation caused very intensive stimulation of anabolism of both precursors, and highest increases in specific activity were detected in the polynucleotides. 3. Uracil and uridine may be utilized in the synthesis of pyrimidine polynucleotides *via* mononucleotides as intermediates or by direct incorporation into the polynucleotide chain. 4. Samples fed at various times of the day show considerable differences in content and metabolism of some of the pyrimidine derivatives, presumably due to the diurnal rhythm of the plants.

In recent years it has been suggested that the amounts of nucleic acids decreased to a considerable extent when leaves were floated on water (Osborne, 1962; Srivastava & Ware, 1965). In our previous work we have noted that excised plants immersed for 8 hr. with their cut off ends in prolonged feeding experiments, have also shown a marked drop in the content of 2′(3′)-UMP, suggesting degradation of RNA (Wasilewska & Reifer, 1967). We therefore assumed that starved plants depleted of nucleic acids may more effectively utilize the precursors of their synthesis than the corresponding non-starved plants. We were particularly interested in the utilization of uracil and uridine, which, as has previously been shown, undergo in plants anabolic changes to pyrimidine nucleotides (Buchowicz, Wasilewska, Witecki & Reifer, 1963; Wasilewska & Reifer, 1967).

MATERIALS AND METHODS

Reagents were purchased from sources as reported previously (Wasilewska & Reifer, 1967).

Experiments were carried out on 6-day-old plants of winter wheat, variety Dańkowska 40 and on 14-day-old pea plants, variety Perła Szlachetna. The plants were excised and starved for 36 or 48 hr., being immersed with their cut off ends in distilled water and kept in darkness at 37°. External moisture was then dried off

with filter paper, the softened ends cut off and the material used for the feeding experiment without delay. Samples of 2 g. were fed with [2-^{14}C]uracil or [2-^{14}C]uridine as described before (Wasilewska & Reifer, 1967). Control samples from the same harvest were cut off immediately prior to feeding. Dry weight was determined on parallel samples. Analytical procedures and determinations of specific activities were carried out as already described (Wasilewska & Reifer, 1967) except that 5′-UMP and 5′-CMP were determined as sums of mono-, di- and triphosphates after hydrolysis in 0.3 N-HCl for 15 min. in a boiling water bath.

RESULTS

Starvation caused a considerable decrease in the dry matter of the pea and wheat plants; after 36 hr. the drop amounted to 19% and after 48 hr. to about 23% (Table 1).

Table 1

Dry matter in starved and non-starved plants

The results of 4 analyses ± S.D. are given.

Plant	Time of starvation (hr.)	Dry matter (%)
Wheat	0	13.37 ± 0.08
	36	10.91 ± 0.11
Pea	0	12.98 ± 0.16
	36	10.57 ± 0.10
	48	10.09 ± 0.10

Table 2 demonstrates the effect of starvation after 36 hr. Each experiment separated in the Table by a horizontal line, represents data from plants of the same harvest. The feeding was carried out in the early morning (7 a.m.). Starvation caused a marked drop in the content of the polynucleotide pyrimidines amounting to about 20%. The content of the mononucleotides remained unchanged. The corresponding results of specific activities of the isolated pyrimidine derivatives demonstrated that starvation stimulated the anabolism of the ^{14}C-labelled substrates. Stimulation with [2-^{14}C]uracil as precursor was the most effective in the polynucleotides, and their specific activities were about 13 times higher than in the non-starved plants. Ribose-5-phosphate in the feeding mixture additionally stimulated the incorporation of the label into pyrimidine derivatives.

The effect of starvation was not limited to stimulation only, as additionally a change in the sequence of the anabolic transformation of uracil has been observed, when compared with the non-starved control plants. In the control plants the highest specific activity was detected in uridine, followed by 5′-UMP, 5′-CMP and the polynucleotides, whereas in the starved plants the specific activity of uridine was

followed by the 2′(3′)-nucleotides, which showed distinctly higher specific activities than their 5′-counterparts. Very high specific activities were detected in the cytidylic nucleotides with label nearly as high as in the uridylic compounds, an observation never before made in our previous work (Wasilewska & Reifer, 1967). This applied to the controls as well as to the starved plants. It is not unlikely that the early hours in which the experiments were carried out may have had some bearing upon the results, as in the work reported previously the plants were fed in the early afternoon. The possible effect of the diurnal rhythm of plants will be discussed later.

Similar effects of starvation upon stimulation of anabolic changes were also observed when uridine was used as the precursor of pyrimidine nucleotides. The specific activities of the isolated derivatives show a 2-3 fold stimulation in the case of mononucleotides and a 8-10 fold stimulation in the case of the polynucleotides. In starved plants the specific activities of 5′-nucleotides were only 1.5 - 2 times higher than those of the polynucleotides, whereas in the control plants they were about 7 times as high. Similarly as when uracil was used as precursor, control and starved plants have shown very effective incorporation of the label into the cytidylic derivatives.

Table 3 shows results with time of starvation extended to 48 hr. As may be seen, additional 12 hr. of starvation have caused a very distinct drop in the intensity of uracil anabolism into polynucleotides, when compared with the samples starved for 36 hr.

The two samples of pea plants used differed considerably in the content of endogenous uridine. The level of uridine in samples of expt. I carried out at 7 p.m. was very low, and a changed sequence of incorporation of the uracil label was observed. The highest specific activity was detected in 5′-UMP; in the non-starved plants it was 2.5 times higher than the label of uridine, and in the starved plants 4 - 6 times higher. Although the stimulatory effect of starvation is marked, it must be pointed out that the low level of uridine was detected in the control plants as well. Similar results on non-starved plants were reported previously for experiments also carried out in the evening (Wasilewska & Reifer, 1967). Experiment II carried out in the early afternoon confirmed that prolonged periods of starvation are detrimental to the incorporation of uracil into polynucleotides. Starved plants fed with [2-^{14}C]uracil in presence of $K_4P_2O_7$ have shown a suppression of incorporation of the label into the pyrimidine derivatives, similar to that observed in non-starved plants (Wasilewska & Reifer, 1967). Feeding with [2-^{14}C]uracil in presence of non-radioactive 5′-UMP caused a considerable drop in specific activity of this mononucleotide as well as of the polynucleotides, with the content of 5′-UMP remaining unaltered.

DISCUSSION

The reported data indicate that a period of 36 hr. of starvation is sufficient to observe the process of degradation of RNA. Owing to the depletion of nucleic acids

Table 2

Effect of 36 *hr. starvation of plants upon the metabolism of uracil and uridine*

The feeding was carried out at 7 a.m. Specific activity: uracil 24 000 counts/sec./μmole; uridine 37 000 counts/sec./μmole. 5′-UMP and 5′-CMP represent the sum of mono-, di- and triphosphates. The amounts of pyrimidine derivatives are expressed as μmoles per gram of fresh tissue. Time of feeding: expts. I and II, 4 hr.; expts. III and IV, 2 hr.

Exp. no.	Plant	Time of starvation (hr.)	Feeding mixture		Intake (counts/sec./g.)	Acid-soluble fraction								Acid-insoluble fraction			
						Uracil		Uridine		5′-UMP		5′-CMP		2′(3′)-UMP		2′(3′)-CMP	
			labelled substrate (mM)	addition (mM)		μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole
I	Wheat	0	[2-^{14}C]Uracil, 1	KH_2PO_4, 10	10 065	0.103	8949	0.202	1527	0.104	51	0.076	50	0.586	21	0.551	19
		36	[2-^{14}C]Uracil, 1	KH_2PO_4, 10	10 462	0.080	7910	0.192	2362	0.099	175	0.067	167	0.438	279	0.422	258
		36	[2-^{14}C]Uracil, 1	KH_2PO_4, 10 +R-5-P, 10	9 860	0.077	7346	0.202	2447	0.096	220	0.070	170	0.454	317	0.439	275
II	Pea	0	[2-^{14}C]Uracil, 1	KH_2PO_4, 10	10 210	0.247	3292	0.521	443	0.175	68	0.140	56	0.950	18	0.941	17
		36	[2-^{14}C]Uracil, 1	KH_2PO_4, 10	10 845	0.188	3357	0.414	835	0.168	187	0.129	110	0.734	242	0.765	218
		36	[2-^{14}C]Uracil, 1	KH_2PO_4, 10 +R-5-P, 10	9 975	0.190	3180	0.422	907	0.171	198	0.132	107	0.755	273	0.776	248
III	Wheat	0	[2-^{14}C]Uridine, 0.65	KH_2PO_4, 10	9 092	0.054	647	0.248	11828	0.088	405	0.063	281	0.543	73	0.542	39
		36	[2-^{14}C]Uridine, 0.65	KH_2PO_4, 10	9 530	0.037	984	0.240	9258	0.085	998	0.069	790	0.448	667	0.429	438
IV	Pea	0	[2-^{14}C]Uridine, 0.65	KH_2PO_4, 10	10 116	0.189	270	0.574	5043	0.161	581	0.131	371	0.936	71	0.880	41
		36	[2-^{14}C]Uridine, 0.65	KH_2PO_4, 10	10 590	0.156	292	0.472	3865	0.164	1024	0.124	1047	0.702	544	0.706	333

Table 3

Effect of 48 *hr. starvation of pea plants upon uracil anabolism*

Specific activity of uracil 24 000 counts/sec./μmole. Time of feeding 2 hr. Expt. I was carried out at 7 p.m., expt. II at 2 p.m. 5′-UMP and 5′-CMP represent the sum of mono-, di- and triphosphates. The amounts of pyrimidine derivatives are expressed as μmoles per gram of fresh tissue.

Exp. no.	Starvation (hr.)	Feeding mixture		Intake (counts/sec./g.)	Acid-soluble fraction								Acid-insoluble fraction			
					Uracil		Uridine		5′-UMP		5′-CMP		2′(3′)-UMP		2′(3′)-CMP	
		labelled substrate (mM)	addition (mM)		μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole	μmole/g.	counts/sec./μmole
I	0	[2-^{14}C]Uracil, 0.5	KH_2PO_4, 10	3722	0.191	5695	0.072	110	0.176	279	0.131	23	1.748	9.6	1.520	4.4
	48	[2-^{14}C]Uracil, 0.5	KH_2PO_4, 10	3740	0.157	6257	0.065	162	0.179	658	0.126	43	1.278	17.6	1.118	11.0
	48	[2-^{14}C]Uracil, 0.5	KH_2PO_4, 10	3075	0.154	6193	0.069	154	0.172	866	0.138	61	1.303	24.1	1.133	13.3
II	0	Physiol. control		—	0.176	—	0.432	—	0.176	—	0.143	—	1.266	—	1.330	—
	48	[2-^{14}C]Uracil, 0.5	—	3150	0.131	8018	0.286	1085	0.171	47	0.141	26	0.747	5.3	0.810	2.8
	48	[2-^{14}C]Uracil, 0.5	KH_2PO_4, 10	2858	0.138	7340	0.288	1238	0.166	56	0.145	26	0.736	5.4	0.885	2.8
	48	[2-^{14}C]Uracil, 0.5	$K_4P_2O_7$, 10	2335	0.138	7947	0.313	784	0.169	35	0.139	18	0.802	2.4	0.901	1.6
	48	[2-^{14}C]Uracil, 0.5	R-5-P, 10	2375	0.130	6182	0.310	1164	0.169	61	0.142	27	0.777	5.8	0.834	2.8
	48	[2-^{14}C]Uracil, 0.5	KH_2PO_4, 10 +5′-UMP, 5	3130	0.252	7026	0.900	126	0.159	24	0.134	26	0.782	0.8	0.840	0.8

there is an increased demand for RNA precursors as witnessed by the intensity of transformation of [2-^{14}C]uracil as well as [2-^{14}C]uridine.

The marked stimulation of the incorporation of both precursors, particularly into the polynucleotides, would prove that 36 hr. of starvation did not to any large extent contribute to the destruction of the enzymes catalysing this process. However, a period of 48 hr. of starvation appeared to be detrimental to the biological properties of the plant material as shown by the rapid decline in the rate of utilization of the precursors used.

Srivastava & Ware (1965) have determined the RNA content of excised barley leaves floated for 7 days on water at 24 hr. intervals and found a rapid decrease of RNA between 24 and 48 hr. They have also shown that incorporation of ^{32}P into RNA declined by 70% in plants starved for 4 days when compared with fresh non-starved plants. These results seem to corroborate our findings, i.e. that prolonged starvation periods alter the plant material and that optimal time for work on stimulation would be 36 hr. or less. After 36 hr. of starvation the incorporation of the uracil label into polynucleotides was 15 times higher than in non-starved plants and unexpectedly the 2′(3′)-nucleotides have shown twice as high specific activity as the 5′-mononucleotides. This observation appears to revive older views that free bases may be directly incorporated into RNA (cf. Brown & Roll, 1955). However, not having been supported by experimental data, they were later discarded as polynucleotide phosphorylase and RNA polymerase were discovered with the di- and triphosphate of nucleosides as exclusive substrates. Yet in view of the reported facts, the synthesis of polynucleotide pyrimidines with the exclusion of intervening mononucleotides still remains open.

In earlier work on uracil anabolism in wheat plants, we found radioactivity in the polynucleotides and were unable to detect the label in the mononucleotides, when low concentrations of the radioactive substrate were employed (Buchowicz *et al.*, 1963). Thus it would appear that direct incorporation of uracil into polynucleotides is possible also in non-conditioned plants, but the process is difficult to detect owing to dominating pyrimidine synthesis of RNA *via* the mononucleotide intermediates.

Buchowicz (1963) has obtained similar results on wheat plant homogenates incubated with [2-^{14}C]uracil and Midgley (1963) has found that [^{14}C]uracil is incorporated to *E. coli* tRNA with the omission of the intracellular mononucleotide pool. He suggested that tRNA is synthesized from a "private" pool of precursors supplied from degradation products of mRNA.

Uridine may also be utilized in starved plants directly in the synthesis of polynucleotide pyrimidines without the intervening step of phosphorylation into 5′-UMP. This view seems to be supported by the ratio of specific activities between 2′(3′)-UMP and 5′-UMP. For example in starved plants of wheat the ratio is 1 : 1.5 and in the controls 1 : 6, which may suggest that the rate of incorporation of uridine in starved plants is 4 times more efficient than in the controls. Results shown in Table 2

indicate that under our experimental conditions uracil as well as uridine were equally effective precursors of cytidylic and uridylic nucleotides. Very high specific activities in 5′-CMP and 2′(3′)-CMP were detected particularly when uracil was the precursor. It is too early to speculate upon the process of amination, but attention must be drawn to amination other than that of UTP (Wasilewska & Reifer, 1967). High specific activities of 2′(3′)-CMP suggest that amination of UMP already incorporated into the polynucleotide chain may be considered, similarly as reported for the change of UMP into ribothymidylic acid (Comb & Katz, 1964).

Feeding of plants reported in this paper was carried out at various times of the day: 7 a.m., 2 p.m., 7 p.m. Within these three groups differences were noted in the endogenous content of some of the derivatives as well as in the intensity and direction of the observed processes. Plants that were fed in the early morning have shown intense incorporation of the label from uracil and uridine into CMP of the mono- and polynucleotide series. Plants fed in the evening were marked by a low uridine and high polynucleotide content and have shown a changed sequence of incorporation of the uracil label when compared with plants fed in the afternoon. It may be mentioned that starved plants kept for 36 and 48 hr. in the dark have retained fully the rhythm of the control plants. Clearly these observations will have to be confirmed in a series of additional experiments, but it does appear that they are somehow connected with the diurnal rhythm of the plant which is independent of introduced periods of light and darkness. Attempts to establish the role of nucleic acids in the mechanism of the diurnal rhythm have not led to conclusive results. So for instance Ehret (according to Bünning,1964) obtained in *Paramaecium* some indication concerning small diurnal changes in the metabolism of nucleic acids. Hastings (1960) believes that only a small fraction of the nucleic acids participates as causative agent of the diurnal rhythm and Karakashian & Hastings (1962), who have observed inhibition of the diurnal rhythm of photosynthesis in *Gonyaulax* in the presence of actinomycin C, suppose that this may be mRNA.

REFERENCES

Brown G.B. & Roll P.M. (1955). In *The Nucleic Acids* (E. Chargaff & J. N. Davidson, eds.) vol. 2, p. 341, Acad. Press, New York.

Buchowicz J. (1963). *Acta Biochim. Polon.* **10**, 301.

Buchowicz J., Wasilewska L.D., Witecki J. & Reifer I. (1963). *Acta Biochim. Polon.* **10**, 67.

Bünning E. (1964). *The Physiological Clock*. Springer-Verlag, Berlin - Göttingen - Heidelberg.

Comb D.G. & Katz S. (1964). *J. Mol. Biol.* **8**, 790.

Hastings J.W. (1960). *Cold. Spr. Harb. Symp. quant. Biol.* **25**, 131.

Karakashian M.W. & Hastings J.W. (1962). *Proc. Natl. Acad. Sci. U.S.* **48**, 2130.

Midgley J.E.M. (1963). *Biochim. Biophys. Acta* **68**, 354.

Osborne D.J. (1962). *Plant Physiol.* **37**, 595.

Srivastava B.J.S. & Ware G. (1965). *Plant Physiol.* **40**, 62.

Wasilewska L.D. & Reifer I. (1967). *Acta Biochim. Polon.* **14**, 41.

WPŁYW GŁODZENIA NA SYNTEZĘ NUKLEOTYDÓW PIRYMIDYNOWYCH U ROŚLIN

Streszczenie

1. Porównywano szybkość włączania [2-^{14}C]uracylu i [2-^{14}C]urydyny w nukleotydy pirymidynowe w roślinach głodzonych i niegłodzonych.

2. Głodzenie powoduje znaczny wzrost anabolicznych przemian obu prekursorów; największy wzrost wbudowywania znaku wykazano w pirymidynach frakcji polinukleotydowej.

3. Wykorzystanie uracylu i urydyny do syntezy polinukleotydów może zachodzić poprzez wolne nukleotydy lub przez bezpośrednie wbudowywanie zasady lub nukleozydu.

4. Próby pobrane do doświadczeń w różnych porach dnia wykazywały znaczne różnice w zawartości i metabolizmie niektórych pochodnych pirymidynowych, co może się wiązać z rytmem dobowym roślin.

Received 6 May, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

MARIA M. JEŻEWSKA, B. GORZKOWSKI and TERESA SAWICKA

THE STRUCTURE OF URIC ACID RIBOSIDE AND CHANGES IN ITS CONTENT IN MOTHS

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

1. The uric acid riboside was isolated from *Bombyx mori* pupae and identified as 3-D-ribosyl uric acid. 2. Changes in the content of uric acid and its riboside were determined in the course of metamorphosis of three species of moths.

Heller & Jeżewska (1960) isolated the uric acid riboside from *Sphingidae* moths. The absorption spectrum of this compound in water and 0.1 N-NaOH had only one maximum between 290 and 300 mμ and in 0.1 N-HCl also a shoulder at 225 - 235 mμ; the spectrum was the same as that of the uric acid riboside isolated from ox blood. As the riboside from ox blood was thought initially to be 9-ribosyl uric acid (Falconner & Gulland, 1939) we assumed the same structure for the compound from *Sphingidae* moths. However, it has been established later (Forrest, Hatfield & Lagowski, 1961; Hatfield, Rinehart & Forrest, 1963; Lohrmann, Lagowski & Forrest, 1964) that the compound from ox blood is 3-D-ribosyl uric acid.

In the present work, the position of ribose in the uric acid riboside isolated from pupae of *Bombyx mori* was established by comparison with the data for synthetic 3-D-ribosyl uric acid. Also the changes in uric acid and uric acid riboside concentration during metamorphosis were examined in three species of moths.

Isolation and structure of uric acid riboside

Ten-day-old pupae of *Bombyx mori* were bled, the soft parts were freed from culticle and freeze-dried. The obtained material, 3 g., was partly delipidated by treatment with acetone - chloroform mixture (5 : 1, by vol.) and then extracted twice with 150 ml. of cold ethanol - water mixture (2 : 1, by vol.). After standing in a refrigerator for 20 min., the pooled ethanolic extracts were centrifuged at 6 000 rev./min., the supernatant evaporated to a small volume in a stream of cold air and then applied to a column of Dowex 1 X8, formate form (200 - 400 mesh, 25×0.5 cm.). The column was washed with water and eluted with a gradient of

formic acid increasing in concentration from 0 to 0.04 N. Fractions of 4 ml. were collected by Unipan automatic fraction collector and extinction was measured at 260, 275 and 280 mμ in Unicam spectrophotometer. The uric acid riboside left the column ahead of the uric acid (Fig. 1). The riboside fractions pooled from

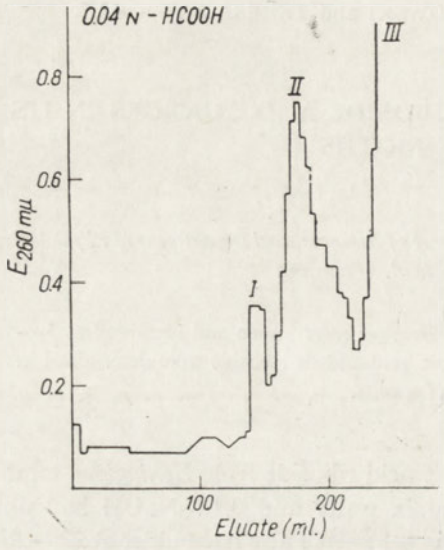


Fig. 1. The diagram of the uric acid riboside gradient elution from the column of Dowex 1X8, formate form (200 - 400 mesh). Fractions: *I*, non-identified substance; *II*, uric acid riboside; *III*, uric acid.

several experiments were freeze-dried and rechromatographed on the column as described above. Then the fractions which had the E_{280}/E_{260} ratio of 2.90 were pooled and freeze-dried.

The freeze-dried material was dissolved in a small volume of water and submitted to chromatography on Whatman no. 3 paper. Three solvents were used: *n*-propanol - water (7 : 3, by vol.); *n*-propanol - 2% NH_3 (3 : 2, by vol.); and 4% aqueous solution of sodium citrate (Hatfield, Greenland, Stewart & Wyngaarden, 1964). In all of them a single spot revealed by ultraviolet light (filter 260 mμ) had the same R_F as the spot of the authentic 3-D-ribosyl uric acid. The spot was cut out and eluted with 0.1 N-HCl. Absorption spectra of the eluate at different pH values were determined (Fig. 2A). There was a good agreement between these spectra and the spectra of the synthetic 3-D-ribosyl uric acid (Fig. 2B), as well as with the data published in the literature (Forrest *et al.*, 1961; Hatfield *et al.*, 1963; Lohrmann *et al.*, 1964). for the compound isolated from ox blood and identified as 3-D-ribosyl uric acid. These spectra differ markedly from those for 9-D-ribosyl uric acid (Holmes & Robins, 1965).

The spectrum of synthetic 3-D-ribosyl uric acid and that of the compound from *Bombyx mori* had a small maximum of absorption at 233 mμ. Instead of this maximum the spectrum of uric acid riboside isolated previously (Heller & Jeżewska, 1960) from *Sphingidae* moths and from ox blood had a shoulder in the region of 225 - 235

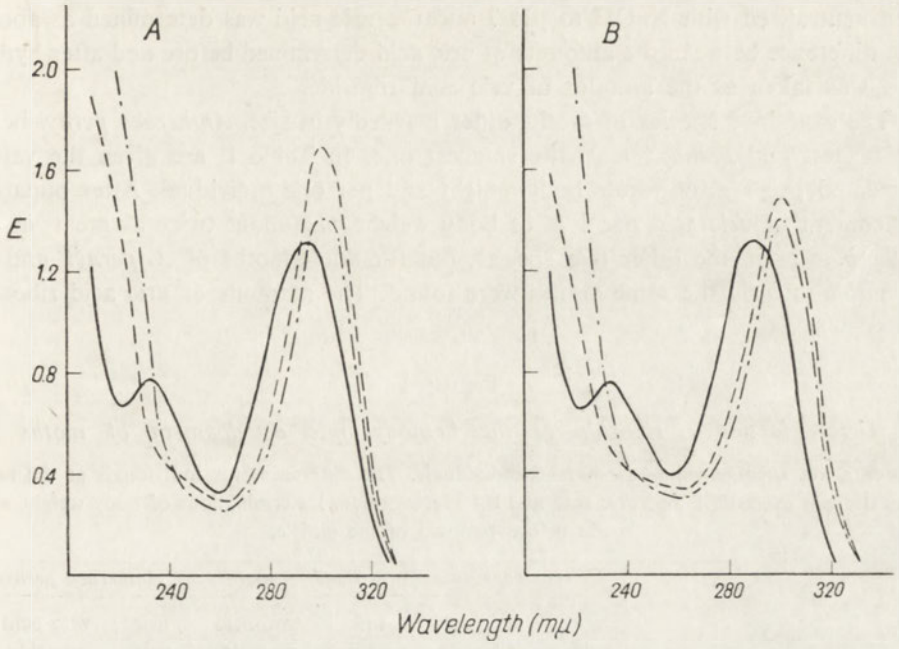


Fig. 2. The absorption spectra of (*A*), uric acid riboside from *Bombyx mori* and (*B*), synthetic 3-D-ribosyl uric acid at (——), pH 2; (— — —), pH 8; and (—·—), pH 12.

mμ. This difference was probably due to impurities of those, not so highly purified, preparations. Probably the compound isolated from *Sphingidae* moths, similarly as that from *Bombyx mori*, also is 3-D-ribosyl uric acid.

Changes in the content of uric acid and its riboside during metamorphosis

The experiments were carried out on three species of moths: *Celerio euphorbiae*, *Bombyx mori* and *Antheraea pernyi*[1], running or spinning larvae in the Vth instar, prepupae, pupae, and adult moths before the deposition of excreta, being used. After removal of the cuticulum the animals were homogenized in a Potter-Elvehjem homogenizer with 10-fold volume of 0.2 M-borate buffer, pH 10. The homogenates were boiled for 1 - 2 min. and the precipitated protein centrifuged off. From the supernatant, the remaining protein was removed with chloroform after Sevag, Lackmann & Smolens (1938). In one part of the protein-free extract, uric acid was determined by the colorimetric method of Caraway (1955), directly and after incubation with uricase obtained according to Leone (1953). The difference between these two values was taken as the amount of uric acid. The second part of the protein-free extract was acidified with conc. HCl to a concentration of 2 N and then heated in a boiling water bath for 6 hr. to hydrolyse the uric acid riboside. The sample was

[1] Preliminary data have been reported (Gorzkowski & Jeżewska, 1963).

then neutralized with NaOH to pH 7 and the uric acid was determined as above. The difference between the amounts of uric acid determined before and after hydrolysis was taken as the amount of uric acid riboside.

The examined species of moths differ markedly in size, *Antheraea pernyi* being the biggest and *Bombyx mori* the smallest one. In Table 1 are given the values calculated per 1 g. of whole body weight and per one individual. After pupation the content of uric acid per 1 g. of body weight was about twice as great in *C. euphorbiae* as in the other two species, but for adult moths of *A. pernyi* and *C. euphorbiae* nearly the same values were found. The amounts of uric acid riboside

Table 1

Uric acid and its riboside in the homodynamic development of moths

The data are average values from 3 - 5 individuals. The differences for individuals at the same stage did not exceed 1.5 mg. uric acid and 0.1 mg. riboside. Determinations of body weight were made before removal of the cuticle.

Animals	*Celerio euphorbiae*		*Bombyx mori*		*Antheraea pernyi*	
	uric acid	uric acid riboside	uric acid	uric acid riboside	uric acid	uric acid riboside
	(mg./g. body wt.)					
Larvae in Vth instar (running or spinning)	5.5	traces	1.0	0.04	—	—
Prepupae						
recent	7.7	traces	2.2	0.06	—	—
before pupation	8.9	traces	—	—	—	—
Pupae (age, days)						
1 - 2	12.0	0.09	4.9	0.11	6.5	0.9
4 - 5	13.1	0.10	6.5	0.28	—	—
7 - 8	15.5	0.23	8.3	0.50	—	—
10 - 11	18.2	0.49	8.7	0.55	—	—
13 - 14	19.5	0.56	eclosion		16.0	1.2
Adults before deposition of excreta	20.0	0.75	8.7	0.66	22.7	3.7
	(mg./individual)					
Larvae in Vth instar (running or spinning)	15.1	traces	3.6	0.13	—	—
Prepupae						
recent	20.2	traces	4.1	0.12	—	—
before pupation	24.2	traces	—	—	—	—
Pupae (age, days)						
1 - 2	24.4	0.17	7.7	0.17	55.3	7.5
4 - 5	28.8	0.23	9.5	0.41	—	—
7 - 8	32.2	0.48	10.4	0.62	—	—
10 - 11	34.5	0.94	11.3	0.71	—	—
13 - 14	33.2	0.94	eclosion		115.7	8.6
Adults before deposition of excreta	34.0	1.28	10.8	0.82	127.1	20.9

were very small in comparison with those of uric acid. Similar values were found for *C. euphorbiae* and *B. mori* at all stages of metamorphosis, the values for *A. pernyi* being always much higher.

As the insects lose weight during metamorphosis, the changes occurring in its course are much more evident when the values are given per one individual. In *C. euphorbiae* and *B. mori* the content of uric acid increased about twofold since the end of Vth instar to the middle of pupal life and then remained constant till the eclosion. On the contrary, there was a steady increase in uric acid riboside till the end of metamorphosis.

Previously (Heller & Jeżewska, 1960) it has been suggested that in insects the uric acid riboside might play some role in the transfer of uric acid. The continuous increase in the content of the riboside during the metamorphosis observed in the present work, is consistent with this supposition.

The authors are indebted to Prof. Dr. D. Shugar for the gift of synthetic 3-D-ribosyl uric acid.

REFERENCES

Caraway W.T. (1955). *Amer. J. Clin. Path.* **25**, 840.
Falconner R. & Gulland J.M. (1939). *J. Chem. Soc.* 1369.
Forrest H.S., Hatfield D. & Lagowski J.M. (1961). *J. Chem. Soc.* 963.
Gorzkowski B. & Jeżewska M.M. (1963). *I Krajowy Kongres Biochemii Łódź, 4 - 7 września 1963* (*Polska*). Streszczenia prac, str. 195.
Hatfield D., Greenland R.A., Stewart H.L. & Wyngaarden J.B. (1964). *Biochim. Biophys. Acta* **91** 163.
Hatfield D., Rinehart R.R. & Forrest H.S. (1963). *J. Chem. Soc.* 889.
Heller J. & Jeżewska M.M. (1960). *Acta Biochim. Polon.* **7**, 469.
Holmes R.E. & Robins R.K. (1965). *J. Amer. Chem. Soc.* **87**, 1772.
Leone E. (1953). *Biochem. J.* **54**, 393.
Lohrmann R., Lagowski J.M. & Forrest H.S. (1964). *J. Chem. Soc.* 451.
Sevag M.G., Lackmann D.B. & Smolens J. (1938). *J. Biol. Chem.* **124**, 425.

STRUKTURA RYBOZYDU KWASU MOCZOWEGO I ZMIANY JEGO ILOŚCI PODCZAS METAMORFOZY MOTYLI

Streszczenie

1. Z poczwarek jedwabnika *Bombyx mori* wyizolowano rybozyd kwasu moczowego i ustalono, że jest to kwas 3-D-rybozylomoczowy.

2. Oznaczono również zmiany ilości kwasu moczowego i jego rybozydu podczas metamorfozy u trzech gatunków motyli.

Received 16 May, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

J. BUCHOWICZ and I. REIFER

DIRECT INCORPORATION OF [2-^{14}C]URACIL INTO POLYNUCLEOTIDES IN ACETONE-DRIED *LUPINUS ANGUSTIFOLIUS*

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

1. The incorporation of [2-^{14}C]uracil into the pyrimidine moiety of the polynucleotides in lupin acetone-dried powder was observed. Most of radioactivity of the acid-insoluble product was recovered after acid hydrolysis in 2′(3′)-UMP; some radioactivity was also detected in 2′(3′)-CMP. 2. No radioactive intermediates were found in the acid-soluble fraction. ATP, uridine and 5′-UMP caused no changes in the rate of uracil incorporation. 3. The possibility of direct binding of free bases to a polyribophosphate backbone, as a way for biosynthesis of some fraction of cellular RNA, was discussed.

It is now well established that only di- and tri-phosphonucleosides can be direct donors of each nucleotide unit for enzymic synthesis of the polynucleotide chain. However, it has also been shown that *in vivo* simple low-molecular precursors can be incorporated with much higher intensity into nucleic acids than into mononucleotides. This apparent contradiction has been explained in two different ways: as the effect of cell compartmentation (McCarthy & Britten, 1962; Kandaswamy & Henderson, 1962; Midgley, 1963) or direct binding of simple precursors, e.g. uracil, to a macromolecular acceptor (Buchowicz, Wasilewska, Witecki & Reifer, 1963; Buchowicz & Reifer, 1964). Studies under conditions which cause destruction of cell structure could unequivocally decide the correctness of either of the two assumptions. Although direct incorporation of [2-^{14}C]uracil into the polynucleotide fraction was previously observed in wheat seedling homogenates (Buchowicz, 1963), yet the low intensity of the process could give some doubts concerning the obtained results. Further attempts were therefore made to find appropriate conditions for efficient incorporation of uracil in a cell-free system.

MATERIALS AND METHODS

Acetone-dried powder was prepared from two-week-old lupin plants (*Lupinus angustifolius*, variety Obornicki) as described by Wilkoszewska, Kleczkowski & Reifer (1961).

For a typical incorporation experiment, 100 mg. of the powder was suspended in 2 ml. of 0.1 M-potassium citrate - phosphate buffer, pH 6.0, and 0.03 μmole of

[2-^{14}C]uracil (specific activity 66 000 counts/sec./μmole) was added to the suspension. The mixture was incubated at 37° under gentle shaking. The incubation was stopped after 1 hr. by cooling in an ice-bath and adding 5 ml. of cold 0.4 N-$HClO_4$, containing non-radioactive uracil, uridine, 5′-UMP and 5′-CMP as carriers (0.2 mM each). In control experiments (zero-time incubation) the radioactive substrate was added to the suspension after cooling, just before deproteinization. All subsequent operations were performed at 2 - 4°.

The inactivated mixture was centrifuged at low speed and the acid-insoluble sediment was washed six times with 20 ml. portions of 0.2 N-$HClO_4$, containing non-radioactive uracil (0.1 mM). The supernatant and washings were combined and used for isolation of uridine and pyrimidine mononucleotides by paper chromatography. The washed sediment was hydrolysed with 1 N-HCl at 100° for 1 hr. and the resulting hydrolysate was used for measurements of radioactivity of the crude polynucleotide fraction, as well as for isolation of the pure 2′(3′)-UMP and 2′(3′)-CMP, according to the previously described procedure (Buchowicz, 1963).

Chemicals. [2-^{14}C]Uracil, The Radiochemical Centre, Amersham, Bucks, England; uracil, uridine, 5′-UMP and 5′-CMP, L. Light Co., Colnbrook, England; ATP, disodium salt, Reanal, Budapest, Hungary; yeast RNA, Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

RESULTS

The results of incorporation of [2-^{14}C]uracil into the crude polynucleotide fraction are given in Table 1. The presence of ATP had no stimulatory effect on the incorporation. Neither uridine nor 5′-UMP showed dilution effects on the observed incorporation of [2-^{14}C]uracil. Yeast RNA, at high concentration, slightly decreased the intensity of the process. Practically no incorporation was observed in the control sample, at zero-time incubation.

Only small radioactivity of uridine, 5′-UMP and 5′-CMP of the acid-soluble fraction was detected in the incubated and control samples (Table 2). On the other hand, pyrimidine mononucleotides isolated from the hydrolysate of the acid-insoluble fraction, especially 2′(3′)-UMP, showed high radioactivity.

Uridine, 5′-UMP and 5′-CMP, added as carriers, were isolated from the incubation mixtures with recoveries equal to 85 - 90%. No measurable endogenous quantities of these compounds could be found in the system. 2′(3′)-UMP and 2′(3′)-CMP were isolated from the hydrolysate in quantities equal to 0.71 and 0.67 μmole, respectively, per assay.

DISCUSSION

A cell-free system capable of supporting the incorporation of [2-^{14}C]uracil into the acid-insoluble fraction has been prepared from lupin plants. The radioactive acid-insoluble product was probably a polynucleotide, because most of its radioactivity could be found after hydrolysis in 2′(3′)-UMP and 2′(3′)-CMP.

Table 1

Incorporation of [2-^{14}C]*uracil into the crude polynucleotide fraction by lupin acetone-dried powder*

The system contained 100 mg. of lupin acetone-dried powder, suspended in 2 ml. of 0.1 M-potassium citrate - phosphate buffer, pH 6.0, and 0.03 μmole of [2-^{14}C]uracil of specific activity 66 000 counts/sec./μmole. Incubation was at 37° for 1 hr. Results are from duplicate experiments, differing by not more than 5%.

Addition	Total radioactivity of crude polynucleotide fraction (counts/sec./assay)
None	14.2
None, zero time	0.1
ATP, 1 μmole	14.2
ATP, 10 μmoles	13.8
Uridine, 1 μmole	10.1
5′-UMP, .1 μmole	12.0
Yeast RNA, 5 mg.	15.7
Yeast RNA, 50 mg.	9.2

Table 2

Distribution of radioactivity in pyrimidine derivatives of lupin acetone-dried powder incubated with [2-^{14}C]*uracil*

Incubation conditions as described in Table 1. Paper chromatography was used for separation of pyrimidine nucleotides (Buchowicz, 1963).

Pyrimidine derivatives	Radioactivity (counts/sec.)	
	Incubated sample	Control sample
Acid-soluble fraction		
Uridine	0.26	0.29
5′-UMP	0.30	0.34
5′-CMP	0.43	0.40
Hydrolysate of the crude polynucleotide fraction		
2′(3′)-UMP	9.72	<0.05
2′(3′)-CMP	1.18	<0.05

Studies on the rate of uracil incorporation under different conditions have revealed an unique character of the system. First, the presence of ATP, the most suitable donor of phosphate groups and energy for uracil anabolism, has not stimulated the incorporation. Secondly, neither uridine nor 5′-UMP, the most probable intermediates on the path of uracil incorporation into polynucleotides, have shown dilution effects. And finally, no radioactive intermediary product could be detected in the acid-soluble fraction. Since the system was free of cell structure, small

"private" pools of pyrimidine mononucleotides as non-detectable intermediates, dependent on cell compartmentation (McCarthy & Britten, 1962; Kandaswamy & Henderson, 1962; Midgley, 1963), seem to be excluded.

These observations, together with the previous findings obtained *in vivo* (Buchowicz *et al.*, 1963; Buchowicz & Reifer, 1964) and *in vitro* (Buchowicz, 1963), clearly suggest that uracil can be incorporated into the acid-insoluble fraction by a simple binding to a suitable macromolecular acceptor of polynucleotide-like character. Such an acceptor should consist of a chain of ribose units substituted in carbon 1 by an unknown, easily exchangeable group and linked, similarly to RNA, by 3→5-phosphate diester bonds. Exchange between the unknown group "*x*" and uracil (probably other bases as well) could lead to conversion of the acceptor to a polynucleotide, according to the following equation:

$$(x\text{-phosphoribose unit})_n + n\text{bases} \rightarrow (\text{mononucleotide unit})_n + nx$$

The compounds with the structure corresponding to our hypothetic acceptor occur in nature. Namely, Zamenhof, Leidy, Fitzgerald, Alexander & Chargaff (1953) and Rosenberg & Zamenhof (1961) described a substance consisting of a polyribophosphate chain as it exists in RNA, in which the place of purines and pyrimidines is occupied by D-ribose. The authors pointed out that it is thermodynamically possible for the polyribophosphate to serve as a precursor for RNA biosynthesis, since the energy of the *O*-glycosidic bond is comparable to that of the *N*-glycosidic bond. Indeed, the high degree of the heterogeneity of cellular RNA makes it possible to assume that some of its molecular species may be synthesized by the binding of free bases to a preformed polyribophosphate backbone.

Apart from D-ribose, the phosphate group may be considered as another candidate for group "*x*" of the hypothetic acceptor. This alternative assumption has its origin in the well known, enzyme catalysed, exchange between uracil and phosphate group of ribose-1-phosphate, described for the first time by Paege & Schlenk (1952).

Low but measurable radioactivity of 2′(3′)-CMP, the second pyrimidine product of the polynucleotide hydrolysate, suggests that in our system an amination reaction on the polynucleotide level could also occur.

The decrease of uracil incorporation rate by RNA was rather unexpected, it may be, however, pointed out that inhibition of RNA synthesis by exogenous RNA was observed recently by Fox, Gumport & Weiss (1965).

Thanks are due to Mrs. Anna Kijewska for skilled technical assistance. This work was supported in part by a grant from the United States Department of Agriculture, Agricultural Research Service.

REFERENCES

Buchowicz J. (1963). *Acta Biochim. Polon.* **10**, 301.
Buchowicz J. & Reifer I. (1964). *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **12**, 63.
Buchowicz J., Wasilewska L.D., Witecki J. & Reifer I. (1963). *Acta Biochim. Polon.* **10**, 67.
Fox C.F., Gumport R.I. & Weiss S.B. (1965). *J. Biol. Chem.* **240**, 2101.
Kandaswamy T.S. & Henderson J.F. (1962). *Biochim. Biophys. Acta* **61**, 86.
McCarthy B.J. & Britten R.J. (1962). *Biophys. J.* **2**, 35.
Midgley J.E.M. (1963). *Biochim. Biophys. Acta* **68**, 354.
Paege L.M. & Schlenk F. (1952). *Arch. Biochem. Biophys.* **40**, 42.
Rosenberg E. & Zamenhof S. (1961). *J. Biol. Chem.* **236**, 2845.
Wilkoszewska I., Kleczkowski K. & Reifer I. (1961). *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **9**, 1.
Zamenhof S., Leidy G., Fitzgerald P.L., Alexander H.E. & Chargaff E. (1953). *J. Biol. Chem.* **203**, 695.

BEZPOŚREDNIE WBUDOWYWANIE [2-^{14}C]URACYLU DO POLINUKLEOTYDÓW W PROSZKACH ACETONOWYCH Z *LUPINUS ANGUSTIFOLIUS*

Streszczenie

1. Stwierdzono wbudowywanie [2-^{14}C]uracylu do pirymidyn polinukleotydów w proszkach acetonowych z roślin łubinu. Radioaktywność nierozpuszczalnego w kwasach produktu wykryto, po przeprowadzeniu kwaśnej hydrolizy, głównie w 2′(3′)-UMP; niewielką radioaktywność znaleziono także w 2′(3′)-CMP.

2. Nie wykryto żadnych radioaktywnych produktów pośrednich we frakcji rozpuszczalnej w kwasach. ATP, urydyna i 5′-UMP nie zmieniały szybkości wbudowywania uracylu.

3. Przedyskutowano możliwość bezpośredniego wiązania wolnych zasad przez polirybofosforanowy szkielet jako jednej z dróg biosyntezy komórkowych kwasów rybonukleinowych.

Received 18 May, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

A.R. GILBY*, SILVER S. WYATT and G.R. WYATT

TREHALASES FROM THE COCKROACH, *BLABERUS DISCOIDALIS*: ACTIVATION, SOLUBILIZATION AND PROPERTIES OF THE MUSCLE ENZYME AND SOME PROPERTIES OF THE INTESTINAL ENZYME

Department of Biology, Yale University, New Haven, Connecticut, U.S.A.

This paper is dedicated with respect and affection to Professor Josef Heller on the occasion of his seventieth birthday.

1. The thoracic muscle of various insects contains an enzyme capable of hydrolysing trehalose. In several cockroaches and a grasshopper the enzyme activity of muscle homogenates is elevated several-fold by repeated freezing and thawing, as previously shown for the *cecropia* silkmoth. With muscle of several flies, however, such treatment had no effect on trehalase activity. 2. In the cockroach, *Blaberus discoidalis*, trehalase activity is concentrated in a microsomal fraction prepared by centrifugation at 105 000 *g*. In such preparations, glycosidase activity is specific for trehalose; it is inhibited strongly by sucrose and weakly by glucose and D-2-glucosamine. 3. In *Blaberus* muscle microsomal preparations, trehalase activity is enhanced as a result of treatment with several anionic, cationic and non-ionic detergents, and by incubation with snake venom, as well as by freezing and thawing. Snake venom, either whole or briefly boiled, gave the greatest activation (up to 10-fold). 4. The activated enzyme exhibits maximal activity at pH 6.0; with untreated enzyme, this could not be determined precisely, but appears to be the same. The K_m for trehalose is 3.3 mM for untreated enzyme and 1.7 mM for activated enzyme. 5. Of the detergents, only deoxycholate solubilizes the microsomal trehalase, and aggregation occurs on removal of the detergent. Snake venom yields trehalase in soluble form which gives single peaks on Sephadex G-200 columns and in sucrose gradient centrifugation. The apparent molecular weights from these methods are 80 000 and 63 000, respectively. It is suggested that this discrepancy may be due to bound lipid. 6. *Blaberus discoidalis* midgut tissue yields a distinct trehalase, which is entirely in the soluble fraction of homogenates and has pH optimum about 5.0 and K_m for trehalose 0.5 mM.

The metabolic use of trehalose depends upon its cleavage to glucose by trehalase, a specific hydrolytic enzyme. Because of the ubiquity of trehalose in insects (Wyatt, 1961), the distribution and properties of trehalase in this animal group are of special interest. Two distinct types of trehalase have been demonstrated in insects: a soluble enzyme which, when purified from several different species, had a pH optimum

* Present address: Division of Entomology, Commonwealth Scientific and Research Organization, Canberra, Australia.

in the neighbourhood of pH 5.5 and a substrate K_m near or below 10^{-3} M (Kalf & Rieder, 1958; Friedman, 1960; Saito, 1960), and a structure-bound trehalase with pH optimum somewhat higher (Zebe & McShan, 1959). It has recently been established that both types of trehalase occur in a single insect species (*Hyalophora cecropia*), the soluble enzyme being concentrated in intestinal tissue and the structure-bound enzyme (which is characterized by higher values of both pH optimum and K_m) in muscle (Gussin & Wyatt, 1964, 1965; Wyatt, Gussin & Gilby, 1965). The latter enzyme could be prepared from muscle homogenates by centrifuging at 105 000 *g*, and was shown to manifest a several-fold increase in activity as a result of repeated freezing and thawing or treatment with oleic acid.

After surveying a number of available insect species, we selected the giant cockroach, *Blaberus discoidalis*, for further studies on muscle trehalase. We now report on some means by which the enzyme may be activated and rendered soluble, and some of its kinetic properties, along with a few comparative observations on the soluble intestinal trehalase of the same species. Trehalose-splitting activity in the intestine of *B. discoidalis* has previously been demonstrated, but not characterized, by Ehrhardt & Voss (1962).

MATERIALS AND METHODS

Reagents. Chemicals were of reagent grade. The sodium dodecyl sulphate was a purified product kindly given by Dr. D. E. Goddard, Lever Bros. Ltd. Triton X-100, a mixture of polyethylene glycol alkyl aryl ethers, was given by Rohm and Haas, Inc. Other detergents were commercial products. Sodium laurate was prepared from lauric acid and purified by extended washing with alcohol. Dodecyltrimethylammonium bromide was recrystallized from water.

Venom of the cottonmouth moccasin, *Agkistrodon piscivorus piscivorus*, was purchased in lyophilized form from Ross Allen's Reptile Institute, Inc., Silver Springs, Florida. Phospholipase C (*Clostridium welchii*) was a product of Worthington Biochemical Corpn., Freehold, N.J. Purified *E. coli* alkaline phosphatase (Garen & Levinthal, 1960) was kindly given by Dr. A. Garen. Crystalline ovalbumin was a gift of Dr. G. Taborsky, and bovine serum albumin a commercial product.

[1,1′-^{14}C]Trehalose was prepared by injecting 50 μc of [1-^{14}C]glucose (0.65 mc/m-mole; New England Nuclear Corpn., Boston, Mass.) into each of two mature larvae of *Hyalophora cecropia* and permitting incorporation for 6 hr. at 25°. Blood was collected, deproteinized with $HClO_4$, deionized by ion exchange (Wyatt, Kropf & Carey, 1963), and trehalose was purified by chromatography on Whatman no. 3*MM* filter paper in 1-butanol - acetic acid - water (5 : 1 : 2, by vol.). The product contained about 17% of the injected radioactivity and had a specific activity of 0.07 μc/μmole.

Insects. The main insect used was *Blaberus discoidalis*, which was maintained in the laboratory at 25° and 60% relative humidity on a diet of Purina Rat Chow, "Pablum", and wheat germ. In most experiments, adult males were used not more than two weeks after molting, but similar results were obtained with females. The

other insects listed in Table 3 were adults obtained from cultures in various laboratories.

Assay of trehalase. The assay was that described previously (Gussin & Wyatt, 1965): enzyme was incubated with trehalose (0.02 M) and buffer in a volume of 0.3 ml. at 30° for 30 min., then the reaction was stopped by boiling and glucose oxidase reagent was added. The colour was developed by a further incubation, stopped by adding HCl, and measured at 401 mμ in a Zeiss spectrophotometer. The buffer used for muscle trehalase was generally sodium phosphate (0.03 - 0.06 M in the reaction mixture), at pH 6.8 in earlier experiments and pH 6.2 (at which the enzyme is slightly more active) in later ones. For the intestinal enzyme, sodium acetate, 0.03 M, pH 5.6, was used. An enzyme unit is the amount that catalyses the release of 1 mμmole of glucose per minute.

Protein was determined by the biuret method (Gornall, Bardawill & David, 1949) for total muscle homogenates, or the method of Lowry, Rosebrough, Farr & Randall (1951) for microsome preparations.

Table 1

Preparation of microsomal trehalase from thoracic muscle of Blaberus discoidalis

Two adult male cockroaches of unknown age yielded 376 mg. of muscle. This was homogenized in 10 ml. tris maleate buffer (0.05 M, pH 6.8) and fractionated by differential centrifugation. Each fraction was assayed both without further treatment and after six cycles of freezing and thawing.

Fraction	Total protein (mg.)	Trehalase		
		Activity before freezing and thawing		Activation factor on freezing and thawing
		Total (mμmoles/min.)	Specific (mμmoles/min./mg. protein)	
Homogenate	87	1013	11.7	5.6
Fibrils, nuclei, mitochondria (7 500 *g*, 15 min.)	70	353	5.1	6.2
Microsomes (105 000 *g*, 30 min.)	2.7	460	151	5.2
Supernatant	17	280	16.5	1.06

Preparation of muscle microsomal fraction. Muscle was dissected from the thoraces and femurs of insects anaesthetized with CO_2, which yielded about 200 mg. fresh weight per individual. The muscle was rinsed in ice-cold insect Ringer solution (Stevenson & Wyatt, 1962), blotted, and thoroughly ground in either sodium phosphate or tris maleate buffer, 0.05 M, pH 6.8, in an all-glass Potter-Elvehjem homogenizer. In experiments with whole muscle, the homogenate was used directly. The conditions for preparation of the "microsomal fraction" are outlined in Table 1. For high-speed centrifugation, the no. 40 head in the Spinco model L ultracentrifuge was used. The microsomal pellet was rinsed before resuspension in buffer (generally

either phosphate or tris, as above). No advantage was found in the use of polyvinylpyrrolidone medium (Gussin & Wyatt, 1965), and in its presence some lowering of trehalase activity was observed. The microsomal fraction from *B. discoidalis* muscle could be stored at 3° for at least 7 days without significant change in either trehalase activity or response to activating treatments. In many preparations, the yield of microsomal trehalase (unactivated) generally fell in the range 100 - 200 enzyme units per insect.

Preparation of intestinal trehalase. The midguts from adult cockroaches were rinsed free of contents, and homogenized in distilled water (0.1 - 1 ml. per insect). The homogenate was centrifuged at 105 000 *g* for 30 min., the supernatant was dialysed overnight against distilled water, and any turbidity that formed during the dialysis was removed by further centrifuging. As shown in Table 2, some 90% of the midgut trehalase is in the soluble fraction. Other experiments showed that there is very little trehalase in the crop and hindgut of *B. discoidalis*. In several preparations, the yield of midgut soluble trehalase generally fell in the range 50 - 120 enzyme units per insect.

Table 2

Trehalase in Blaberus discoidalis *intestine*

The midguts of 3 adult insects were homogenized in 3 ml. distilled water and centrifuged as shown. The pellet was re-suspended, and both fractions were assayed for trehalase, in sodium acetate buffer, 0.05 M, pH 5.6.

	Fractions from 105 000 *g*, 60 min.	
	Supernatant	Precipitate
Total protein (mg.)	2.4	6.3
Total trehalase activity (mμmoles/min.)	460	54
Trehalase specific activity (mμmoles/min./mg.)	190	8.6

Activating treatments. Freezing and thawing was effected by immersing a tube of enzyme preparation alternately in a solid CO_2-ethanol bath and in water at room temperature. Six cycles of freezing and thawing were used to achieve maximum activation.

The effects of detergents were tested by adding the detergent to microsomal enzyme preparation and holding it at 25° for 15 min. before proceeding with the trehalase assay. It was shown to be unnecessary to maintain the detergent concentration during the assay, and it was desirable to dilute the mixture before assaying in order to avoid turbidity as a result of precipitation of certain detergents (notably deoxycholate) when acid was added at the end of the assay. With sodium laurate, addition of alcohol with the acid helped to prevent precipitation. In testing the effects of urea, it was found necessary to maintain its concentration during incubation with trehalose, and to correct for the effect of urea on the glucose estimation. After

activation, preparations were tested for solubilization of trehalase by assay of the supernatant after centrifugation at 105 000 *g* for 1 hr.

Estimation of enzyme molecular weight. Sephadex G-200 (bead form) was handled as recommended by Andrews (1964). Columns measured 1.2 cm.×48 cm. and effluent was collected in fractions of 0.8 ml. Sucrose gradient centrifugation was performed according to Martin & Ames (1961). Gradients (5 - 20% sucrose in tris buffer, 0.05 M, pH 7.5, containing KCl, 0.1 M) were centrifuged in the SW-39 rotor of the Spinco Model L ultracentrifuge, and fractions of 0.15 ml. were collected. Ferritin and cytochrome *c*, used as markers, were detected by absorbancy at 408 mμ, ovalbumin and serum albumin by absorbancy at 280 mμ, and enzyme assays were used for trehalase and for alkaline phosphatase (Lowry, 1957). In assaying trehalase from the sucrose gradients, the concentration of trehalose was raised to 0.1 M in order to decrease the inhibition by the sucrose. The following molecular weights were assumed: bovine cytochrome *c*, 12 500; ovalbumin, 45 000; bovine serum albumin, 67 000; *E. coli* alkaline phosphatase, 75 000.

RESULTS

Trehalase in insect muscle; activation by freezing and thawing

The trehalase activities of homogenates of thoracic muscles from a number of insects are set out in Table 3. The enzyme specific activity and the activation shown

Table 3

Trehalase activity of insect muscle homogenates and activation by freezing and thawing

Thoracic muscles from 3 - 20 adult insects were homogenized in phosphate buffer (0.05 M, pH 6.8) and assayed both without further treatment and after six cycles of freezing and thawing. (a), Adults less than 2 days after moult or emergence; (b), adults more than 10 days old. *Phormia sericata* and *Musca domestica* adults were 7 - 8 days old.

Insect	Trehalase specific activity (mμmoles/min./mg. protein)	Activation factor by freeze/thaw
Blattodea		
Blaberus discoidalis (a)	9.0	5.4
(b)	6.7	2.6
Gromphadorhina portentosa	9.3	2.1
Panchlora nivea	29.3	2.7
Orthoptera		
Melanoplus differentialis	14.7	2.7
Lepidoptera		
Hyalophora cecropia (a)	4.5	5.0
Diptera		
Sarcophaga bullata (a)	126.0	0.97
(b)	119.2	0.97
Phormia sericata	118.7	0.98
Musca domestica	48.5	0.96

by *H. cecropia* are similar to those found by Gussin & Wyatt (1965). Of the cockroaches, *Panchlora nivea*, which is a strong natural flier, shows the highest trehalase activity. Muscle from all the insects except the Diptera show the phenomenon of activation by repeated freezing and thawing. The flies show the highest trehalase specific activities, but none of the three species investigated showed any activation by freezing and thawing. In *Sarcophaga bullata*, the trehalase was shown to be structure-bound, sedimenting readily in the centrifuge.

Microsomal trehalase from Blaberus discoidalis *muscle*

The preparation adopted for further experiments on muscle trehalase was the *Blaberus discoidalis* microsomal fraction prepared as described in Table 1. This had approximately ten times the trehalase specific activity, in terms of protein, of comparable preparations from *H. cecropia* (Gussin & Wyatt, 1965). A test of several glucosides as potential substrates indicated that the preparation, like that from *H. cecropia*, exhibits specificity for trehalose (Table 4).

Table 4

Substrate specificity and inhibition of Blaberus *muscle trehalase*

Muscle microsomal preparation, activated with deoxycholate, was incubated with each of the sugars and glycosides shown (0.02 M) with and without trehalose (0.01 M). Results are corrected for blanks with boiled trehalase.

Substance tested	Color yield (E_{401})		Apparent inhibition (%)
	Test substance alone	Substance plus trehalose	
Trehalose	0.443	—	—
D-2-Glucosamine	0.021	0.126	71*
Sucrose	0.009	0.140	68
Maltose	0.064	0.415	6
Methyl-α-D-glucoside	0.010	0.440	0
Salicin	0.004	0.260	41*

* Results unreliable because of inhibition of glucose oxidase by these substances (Gussin & Wyatt, 1965).

Activation by physical treatments. The increase in trehalase activity produced by freezing and thawing of muscle microsomal preparations was maximal after about 4 cycles of treatment, as with the *cecropia* enzyme. The factor of activation was generally greater for the microsomal fraction than for whole muscle homogenate, as previously noted with similar preparations from *H. cecropia* (Gussin & Wyatt, 1965). Freeze-thaw activated enzyme remained fully sedimentable at 105 000 *g*. The trehalase in the supernatant fraction from muscle homogenates was not activatable. Because of the likelihood that the disruptive effect of freezing and thawing on biological membranes results in part from the local concentration of electrolytes

as water freezes out (Lovelock, 1954), we performed several experiments to test the influence of salts on freezing-thawing activation. These gave curiously inconsistent results, but were suggestive that sodium phosphate or other salts do assist the freezing-thawing activation. This question needs further investigation.

Sonic oscillation ("Biosonik II", Bronwill Scientific Corpn., New York; 20 kc.) activated weakly: after 10 sec. oscillation of a microsomal preparation, trehalase activity was doubled and the enzyme remained 90% sedimentable; after 60 sec. treatment, activity had fallen to 1.3× the initial level, but remained 88% in the supernatant after centrifuging.

Attempts to activate the microsomal trehalase by osmotic shock were unsuccessful. The trehalase activities of membrane preparations made in the presence of either 4 M-NaCl or the 7.3% polyvinylpyrrolidone medium of Gussin & Wyatt (1965) and diluted into 20 volumes of distilled water either rapidly or slowly (30 min.) were all the same as controls.

Activation by detergents. The effects of several surface-active compounds on trehalase activity of muscle microsomal preparations are illustrated in Fig. 1. All of the detergents tested activated microsomal trehalase, and the maximum activation attained was consistently higher than that produced by freezing and thawing, the activation factors being about 7 and 4 - 5, respectively. The lower maximal activation achieved with dodecyltrimethylammonium bromide (DTAB) was a characteristic of the particular enzyme preparation used in this experiment, which showed low

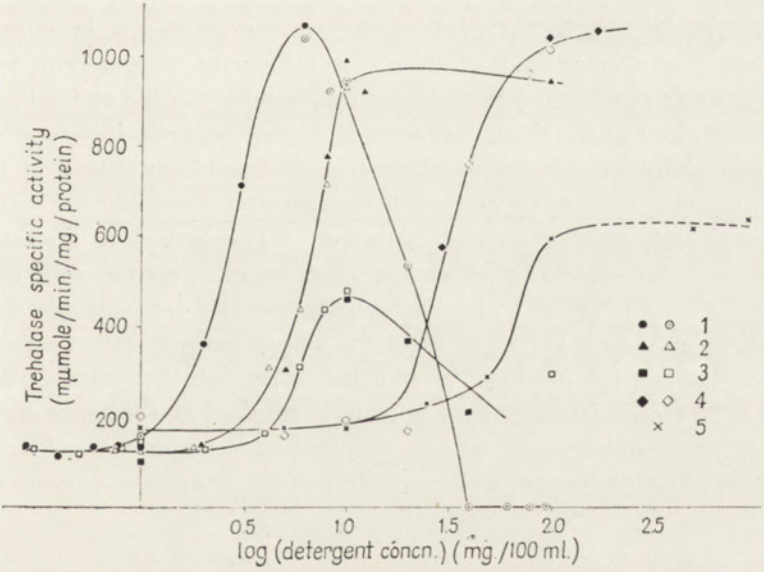


Fig. 1. Effect of detergents on trehalase activity. Muscle microsomal fraction was treated with detergent for 15 min. at 25° before trehalase assay. Open and closed symbols of same shape represent results from separate experiments. 1, SDS; 2, Triton X-100; 3, DTAB; 4, DOC; 5, SL. Abbreviations are as in Table 5.

activation factors when treated with sodium deoxycholate (DOC) and freezing-thawing also. In the experiments with sodium laurate (SL), the preparations showed the normal activation with other detergents, but the higher "concentrations" of SL exceeded the solubility of the soap, so that activation was presumably limited by solubility. Both sodium dodecyl sulphate (SDS) and DTAB inactivate the enzyme at the higher concentrations.

Table 5

Relative efficiency of detergents in activating and solubilizing microsomal trehalase

Microsomal enzyme preparation in sodium phosphate buffer (0.05 M, pH 6.8) was mixed with detergent 15 min. before assay. Percent solubilization refers to the activity remaining in the supernatant after centrifugation at 105 000 *g* for 60 min.

Detergent	Concentration for maximal activation		Efficiency relative to SDS	% Solubilization of trehalase
	mg./100 ml.	mM		
Sodium dodecyl sulphate (SDS)	5	0.17	1.0	5
Dodecyltrimethylammonium bromide (DTAB)	10	0.32	0.5	1
Sodium deoxycholate (DOC)	100	2.4	0.07	96
Sodium laurate (SL)	100	4.5	0.04	2
Triton X-100	10	—	—	11

The efficiencies relative to SDS of the various detergents in terms of the concentrations required to produce maximal activation are given in Table 5, together with the percentages of total trehalase rendered "soluble" by the optimal concentration of each detergent. Of the compounds tested, only DOC was effective in solubilizing. Untreated control microsomal preparations centrifuged in the same way gave no more than a few percent of their enzyme activity in the supernatant.

Effects of other chemicals. Chloroform, added as one drop to a small volume of microsomal suspension as a preservative, was found to activate fully (compared with DOC) during 18 hr. at 2°, but the enzyme remained sedimentable. 1-Butanol, on the other hand, added at 10% by volume to an enzyme preparation in tris maleate buffer, pH 6.8, and left overnight at 2° failed to activate or solubilize. With urea (15 min. at 25°), trehalase activity was scarcely changed at 1 M concentration, but reduced to approximately 30% in 2 M, and inhibited completely in 4 M, 6 M, and 8 M. This inhibition was reversible, since dilution of the urea restored normal trehalase activity.

Activation and solubilization by snake venom. Because of its high phospholipase A activity and effectiveness in disrupting various lipoprotein membranes, snake venom was tested as an activating agent. This activation was as great as, or in some cases up to 30% greater than, that brought about by deoxycholate, so that 9 - 10 fold activation by venom was not unusual. The results of two representative

experiments are shown in Table 6. Snake venom is capable of rendering microsomal trehalase fully soluble; extensive activation, however, can take place without solubilization. Venom that has been briefly heated to boiling and centrifuged to remove the precipitated protein was effective in activating, but less effective than whole venom in solubilizing. Treatment for longer times with heated venom, however, did solubilize. Other experiments showed that activation by venom proceeded somewhat more rapidly at pH 8.5 than at pH 7.4 and that addition of Ca^{2+} (which activates purified phospholipase A) was not essential under our conditions.

Table 6

Activation and solubilization of microsomal trehalase by snake venom

Dried venom of *Agkistrodon p. piscivorus* was dissolved at 10 mg./ml. in tris maleate buffer, 0.1 M, pH 6.0, or tris, 0.1 M, pH 7.4, and portions were heated to 100° for 3 min. and centrifuged. Expt. 1: microsomal preparation (178 μg. protein) was incubated with venom solution in tris buffer, pH 7.6, containing 1 mM-$CaCl_2$, total vol. 0.05 ml., at 30° for 80 min. Expt. 2: microsomal preparation (67 μg. protein) was incubated with venom solution in tris buffer, pH 8.4, containing 0.5 mM-$CaCl_2$, total vol. 0.2 ml., at 30° for 60 min. In both experiments, the reaction mixtures were then chilled, diluted, sampled for assay, centrifuged at 105 000 *g* for 60 min., and re-assayed.

Expt.	Activating treatment	Trehalase activity (units/mg. protein)	Activation factor	Percent solubilized
1	None	78	—	—
	Venom whole (20 μg.)	730	9.3	63
	Venom whole (100 μg.)	766	9.8	93
2	None	142	—	0
	Freezing and thawing 6×	780	5.5	—
	DOC, 0.1%, 25°, 15 min.	828	5.8	17*
	Venom whole (50 μg.)	800	5.6	97
	Venom heated at pH 6.0 (same volume)	918	6.5	80
	Venom heated at pH 7.4 (same volume)	873	6.2	9

* Diluted, bringing the DOC concentration to 0.01%, before centrifuging.

Phospholipase C (from *Cl. welchii*; 0.5 mg./ml. in tris buffer, pH 7.8 at 30° for 60 min.) had virtually no activating effect on microsomal trehalase.

Physical properties of solubilized muscle trehalase. The soluble trehalase obtained by snake venom treatment of *B. discoidalis* muscle microsomal fraction could be precipitated with ammonium sulphate, redissolved, and dialysed with retention of most of its activity. The concentration of ammonium sulphate required to precipitate the enzyme was not precisely determined, but was in the neighbourhood of 60 - 70% of saturation at 0°.

To obtain an estimate of the molecular weight we examined the behaviour of solubilized trehalase on columns of Sephadex G-200 (Andrews, 1964) and in sucrose gradient ultracentrifugation (Martin & Ames, 1961). In both procedures, the venom-

solubilized enzyme gave single symmetrical peaks (Fig. 2) which accounted for a high percentage of the initial trehalase activity. With enzyme solubilized with DOC, on the other hand, only a small fraction of the activity applied was recovered as a peak either from Sephadex columns or from sucrose gradients. Probably, the enzyme was in a form which aggregated upon separation from the detergent.

On the Sephadex columns, the peak of trehalase coincided closely with that of alkaline phosphatase. By plotting elution volume against logarithm of molecular weight for cytochrome *c*, ovalbumin and alkaline phosphatase, the apparent

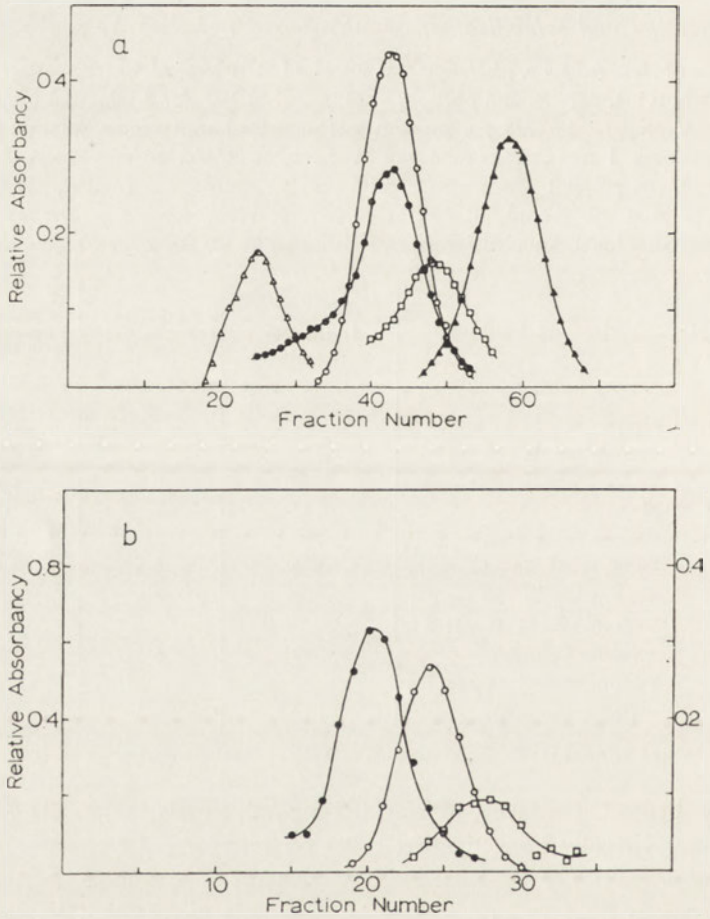


Fig. 2. Molecular weight estimation of muscle trehalase. Enzyme from muscle microsomal fraction was solubilized with snake venom, precipitated with 60% saturated ammonium sulphate and dialysed. (a), Mobility on column of Sephadex G-200 run in tris buffer, 0.05 M, pH 7.5, containing 0.1 M-KCl, at 2°. The symbols, and total amounts applied to the column, are: △, ferritin (1 mg.); ○, trehalase (236 enzyme units); ●, alkaline phosphatase (7.5 μg.); □, ovalbumin (2 mg.); ▲, bovine cytochrome *c* (0.5 mg.). (b), Mobility in sucrose gradient centrifugation at 39 000 rev./min. for 16 hr. Symbols, and amounts applied to the gradient, are: ○, trehalase (118 enzyme units); ●, alkaline phosphatase (2.5 μg.); □, ovalbumin (250 μg.).

molecular weight of venom-solubilized muscle trehalase was estimated as 80 000 (two experiments in good agreement). In gradient centrifugation, trehalase sedimented considerably more slowly than alkaline phosphatase. Since two markers were used on each gradient (alkaline phosphatase and either ovalbumin or serum albumin)

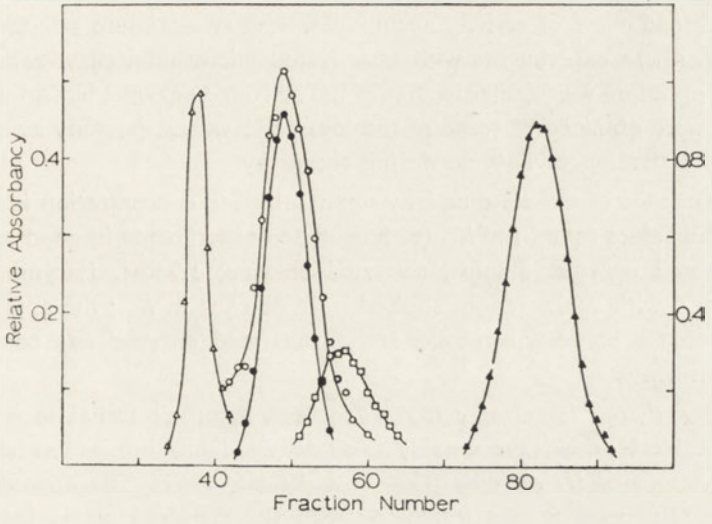


Fig. 3. Molecular weight estimation of intestinal trehalase with Sephadex G-200, run in sodium phosphate buffer, 0.05 M, pH 6.8, at 25°. Symbols, and total amounts applied to the column, are: △ ,ferritin (0.5 mg.); ○ , trehalase (supernatant fraction from midguts, 75 enzyme units); ● , alkaline phosphatase (5 μg.); □, ovalbumin (1 mg.); ▲, horse cytochrome *c* (1 mg.).

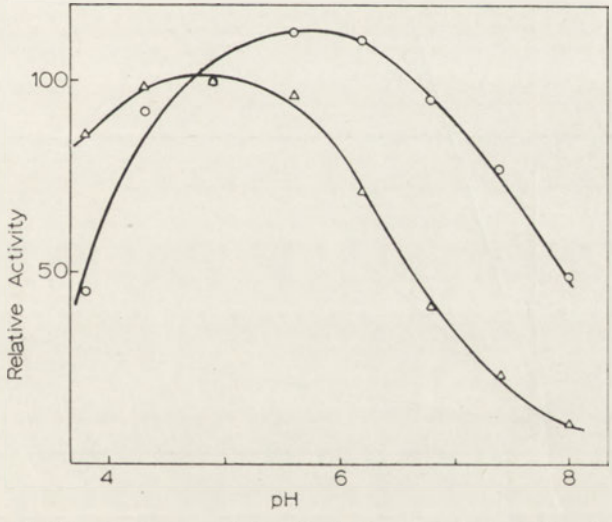


Fig. 4. Effects of pH on trehalase activity. The buffer contained acetic acid, maleic acid and tris, each at 0.06 M, with the pH adjusted with NaOH. Each point is the mean from two separate experiments. ○, Muscle microsomal trehalase, treated with 0.1% DOC; △, midgut trehalase (dialysed supernatant fraction).

molecular weight was estimated from an assumed linear relationship between logarithm of molecular weight and logarithm of distance sedimented from the meniscus. Three experiments with muscle trehalase gave 59 000, 61 000, 69 000 (mean value, 63 000) for the apparent molecular weight.

Kinetic properties. Trehalase from *B. discoidalis* muscle, activated by DOC, exhibited a broad curve of activity against pH, with an optimum at approximately pH 6.0 (Fig. 4). In experiments with unactivated microsomal enzyme it appeared that the pH optimum was similar to that of the activated enzyme, but some aberrant high values were obtained at some of the lower pH values, possibly as a result of spontaneous activation of enzyme during the assay.

The dependence of trehalase activity upon substrate concentration is illustrated in Fig. 5. The values found for K_m (means of two experiments in good agreement) are: unactivated enzyme, 3.3 mM, activated enzyme, 1.7 mM. Enzyme activated with DOC and with snake venom showed no difference in K_m; the approximately twofold difference between activated and unactivated enzyme was confirmed in several experiments.

Inhibitors and stimulators of activity. Cockroach muscle trehalase is inhibited by sucrose (Table 4); this is presumably a competitive inhibition, as has been shown for the enzyme from *H. cecropia* (Gussin & Wyatt, 1965). The apparent strong inhibition by glucosamine was unreliable with the standard assay, because this sugar inhibits glucose oxidase. We, therefore, tested inhibition by measuring the

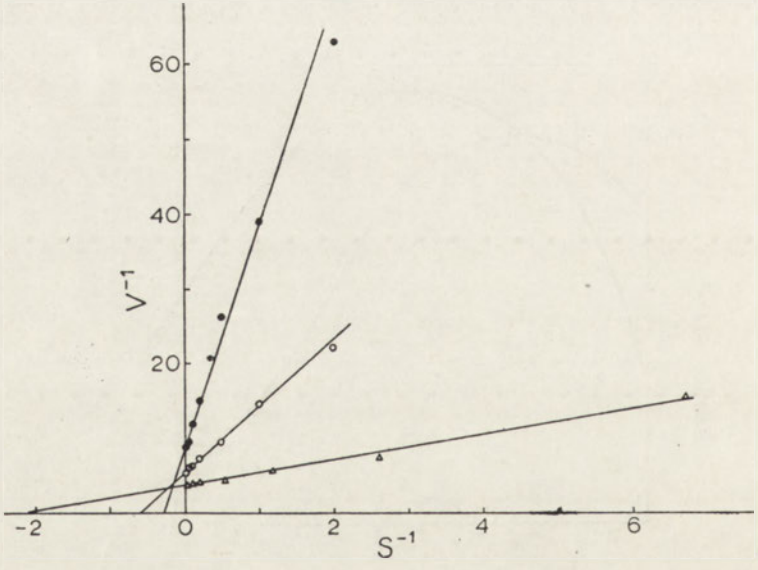


Fig. 5. Relations of reaction velocity to trehalose concentration; double reciprocal plot. S, Trehalose concentration (mM); V, reaction velocity (μmoles of glucose released in 30 min.). ●, Muscle microsomal trehalase (32 μg. protein per tube), not activated; ○, same, treated with 0.1% DOC (8 μg. protein per tube; data from the same experiment); △, midgut trehalase (from a separate experiment).

release of glucose from [^{14}C]trehalose (Table 7). The inhibition by sucrose was confirmed, but glucosamine and glucose at the same molar concentration were found to be only about half as effective as inhibitors.

A number of substances which were of interest because of possible metabolic regulatory roles, or for other reasons, were tested for their effects on trehalase activity. When venom-activated enzyme was assayed in sodium maleate buffer

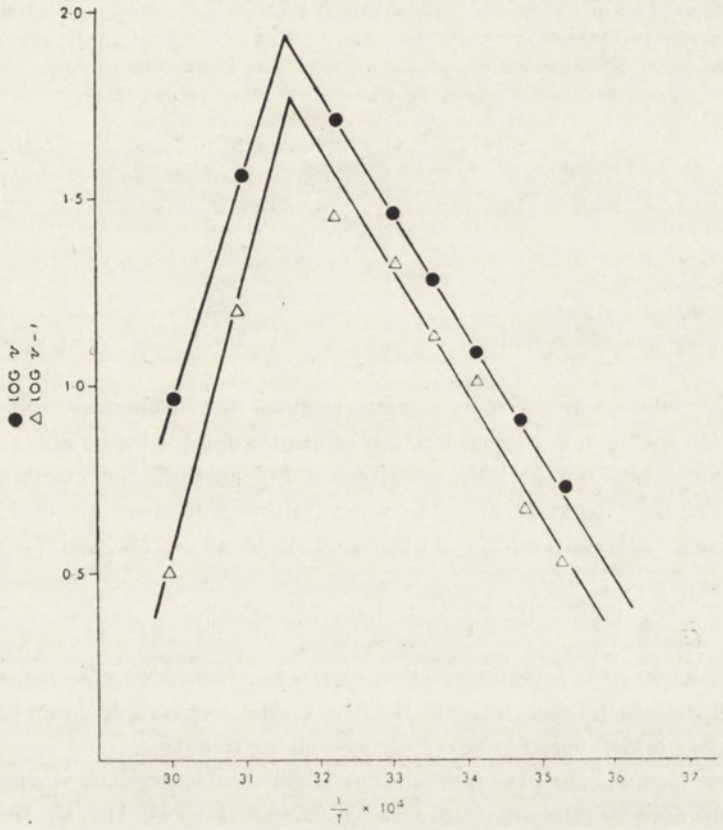


Fig. 6. Effect of temperature on trehalase activity; Arrhenius plot. ●, Muscle microsomal fraction; △, soluble supernatant after treatment of microsomal fraction with 0.1% DOC. *v*, Reaction velocity (arbitrary units).

(0.05 M, pH 6.2), none of the following present during the assay gave more than 20% enhancement or depression of the activity compared with controls: ATP (1 and 5 mM), AMP (1 and 5 mM), glucose-6-phosphate (5 mM), citrate (10 mM), $CaCl_2$ (5 mM), $MgCl_2$ (5 mM), NaCl (0.1 M), Na_2SO_4 (0.05 M), NaH_2AsO_4 (0.05 M). Under these conditions, inorganic phosphate stimulated slightly (about 15% at 0.05 M). In several other experiments, inorganic phosphate appeared to give greater stimulation, but, for reasons which are not understood, this effect was not obtained consistently. Tris-(hydroxymethyl)-aminomethane (0.05 M, adjusted to pH 6.2, added to assays in maleate buffer, 0.03 M, pH 6.3), inhibited about 15%.

Table 7

Inhibition of muscle trehalase by sugars, assayed by radioactivity

Soluble fraction from snake venom-treated muscle microsomes was mixed with [1-^{14}C]trehalose (0.3 μmole containing 11 000 counts/min.) and other sugars as indicated (0.6 μmole) in maleate buffer, 0.03 M, pH 6.3, total vol. 0.03 ml. After 30 min. at 30°, the mixture was heated to 100° for 0.5 min. and centrifuged, and duplicate portions of 0.01 ml. were applied to Whatman no. 3*MM* paper and chromatographed in 1-butanol - acetic acid - water (5 : 1 : 2, by vol.) for 18 hr. Marginal strips carrying markers of glucose and maltose (which migrates at the same rate as trehalose) were cut and sprayed with aniline-diphenylamine reagent (Sigma Chemical Co.). The glucose and trehalose regions of the unsprayed chromatogram were cut, dropped into toluene phosphor and counted in a Packard Tri-Carb liquid scintillation counter.

Reaction	Radioactivity found in glucose (counts/min.)	Inhibition of trehalase (%)
Boiled enzyme	0	—
Standard conditions	3305	—
Sucrose added (0.02 M)	960	71
Glucose added (0.02 M)	2294	31
D-2-Glucosamine added (0.02 M)	2160	35

Effect of temperature. Arrhenius plots derived from trehalase assays over the range 10 - 60° for the microsomal fraction of muscle and for the soluble supernatant after treatment with 0.1% DOC are given in Fig. 6. Both the membrane bound and the solubilized enzymes have the same transition temperature of 42° and the same apparent activation energy (Gibson, 1953) of about 15 Kcal. mole^{-1}.

Soluble trehalase from Blaberus discoidalis *midgut*

A few comparative experiments were performed with the supernatant fraction from *B. discoidalis* midguts (Table 2). This crude preparation contained sucrase and doubtless other digestive enzymes, as well as trehalase.

Kinetic properties. The pH optimum for midgut soluble trehalase is approximately pH 4.8, and activity falls off steeply above pH 5.6 (Fig. 4). The K_m for trehalose is also distinctly different from that of the muscle enzyme (Fig. 5), the mean value from two determinations being 0.5 mM.

Physical properties. Dialysed soluble midgut trehalase was chromatographed with Sephadex columns in the same manner as the solubilized enzyme from muscle (Fig. 3). The trehalase eluted very slightly later than alkaline phosphatase, and its molecular weight is estimated as approximately 70 000. Sucrose gradient analysis was prevented by the sucrase content of the preparation.

DISCUSSION

We have established that *Blaberus discoidalis*, like *Hyalophora cecropia*, possesses two distinct trehalases which are concentrated in muscular and in intestinal tissue, respectively. It seems likely that this is a common situation in insects. In both of

the species examined, the structure-bound trehalase of muscle has a pH optimum about 1.0 unit higher than the soluble trehalase of intestine, and also a higher value of K_m. These parameters are not identical, however, for the corresponding enzymes from the two insects. The muscle trehalases of several Blattodea, Orthoptera and Lepidoptera have also in common the property, first noted by Zebe & McShan (1959), of being activated several-fold by repeated freezing and thawing, but in three species of Diptera, although the thoracic muscle was rich in particle-bound trehalase, the latter was quite unaffected by this treatment. Gussin & Wyatt (1965) have suggested that in *cecropia* the enzyme may be associated with the sarcoplasmic reticulum, a membrane system which is relatively abundant in the synchronous muscle of such insects as moths and cockroaches (Smith, 1962). But, since the asynchronous flight muscle of flies contains very little sarcoplasmic reticulum (Smith, 1962) it is possible that the trehalase is somehow differently located in this muscle[1]. Its failure to be activated may be related to a different structural association. In the present work with cockroach muscle, we designate our preparations as microsomal because they were obtained by centrifugation at 105 000 *g*, but we have not attempted to assess their content of sarcoplasmic reticulum, fragments of sarcolemma, or other structures. The heterogeneity of "microsomal" preparations from different sources has been pointed out by Siekevitz (1963).

Of particular interest is the great increase in trehalase activity when cockroach muscle microsomal preparations are subjected to various physical and chemical treatments. That activation can be brought about by repeated freezing and thawing, and to some extent by sonic oscillation, suggests that physical disruption of membrane structure can expose the enzyme. We attempted to activate by osmotic shock because of the possibility that the enzyme might be located in the inner surfaces of intact microsomal vesicles (Redman, Siekevitz & Palade, 1966), but this was not successful. All of the detergents tested, which included anionic, cationic and non-ionic compounds, were effective in activating to roughly the same extent, although different levels of the different detergents were required for maximal effect. Similarly, chloroform could activate. A potent activating agent was venom of the cottonmouth moccasin, which is known to be rich in phospholipase A capable of attacking lipoproteins (Marinetti, 1965). That the phospholipase is responsible for the effect of venom is supported by the fact that boiled venom is active, for snake venom phospholipase is one of the few enzymes stable in solution at 100° (Slotta, 1960). Different agents caused somewhat different extents of activation, and the accumulated evidence of many experiments indicates the following series: phospholipase A > > detergents > freezing and thawing > sonic oscillation. It seems likely that the microsomal enzyme is embedded in membrane lipoprotein, and that the effectiveness of activating treatments depends upon the completeness with which they dissolve such structure.

---

[1] Since this was written, a paper has appeared by Hansen (1966), who demonstrated that in *Phormia regina* flight muscle the trehalase is 75% particulate and that isolated mitochondria exhibit high specific activity. It is not clear, however, that the microsomal fraction was examined.

Certain mammalian microsomal and mitochondrial enzymes are activated to some extent by some of the agents we have used, but the great extent of activation observed with insect muscle trehalase, and the broad range of effective agents, is more reminiscent of mammalian lysosomal enzymes (Sawant, Desai & Tappel, 1964). The sedimentation properties of our preparations, however, and the ineffectiveness of osmotic shock, preclude their representing lysosomes.

The K_m value found for the fully activated enzyme is approximately half that for untreated microsomes. This difference is most simply accounted for in terms of increased accessibility of substrate to enzyme upon unmasking of the latter. The increase in activity obtainable (up to 10-fold), however, is much greater than could be caused by the change of K_m alone, even if assayed at low substrate levels, and it follows that activating treatments result in exposure of much enzyme which was previously inactive, presumably because of total concealment.

Release of muscle trehalase in soluble form, defined as failure to sediment during 60 min. at 105 000 *g*, required more specific and extensive attack than simple activation. Of the detergents tested, only DOC was effective, and in this case there was evidence of the enzyme re-aggregating upon removal or dilution of the detergent (although activity remained high). The experiments with snake venom also showed that solubilization required more exhaustive treatment than activation. Although venom phospholipase A is reported to be completely stable to brief boiling at pH values below 7 (Slotta, 1960), we found that heated venom possessed decreased solubilizing power when compared with whole venom. This suggests participation of some other component of the venom, possibly a protease. In this connection, it is interesting to note that the lysis of red blood cells by cobra venom involves a "direct lytic factor" as well as phospholipase (Condrea & de Vries, 1964).

The enzyme rendered soluble by the action of snake venom exhibited a single symmetrical peak during Sephadex chromatography or sucrose gradient centrifugation. The apparent molecular weight (by simple comparison with the mobility of marker proteins) was substantially lower by centrifugation (about 63 000) than on the columns (80 000). Migration in gel filtration columns depends fundamentally on the Stokes radius (Siegel & Monty, 1966). Our results indicate that the enzyme has a lower sedimentation constant than would be expected of a simple globular protein of its size. This could result from asymmetry, or from low buoyant density. For an enzyme released from structural lipoprotein, the latter interpretation, as a result of bound lipid, seems the more likely. In the absence of data on the diffusion constant or partial specific volume, more cannot be said. It would be of interest to measure these parameters, and also to examine the portion of the muscle enzyme which occurs naturally in soluble form, in the 105 000 *g* supernatant fraction.

The soluble trehalase from intestinal tissue, when chromatographed on Sephadex columns, gave a peak close to, but possibly of slightly lower molecular weight than, that of the muscle enzyme. It is unfortunate that the sedimentation behaviour of the gut enzyme could not be measured with the techniques and the crude preparations at hand, because of the sucrase activity.

It has been surmized (Gussin & Wyatt, 1965) that the activation phenomenon may be related to the biological regulation of use of trehalose by insect muscle. Our results cast some doubt on this. Activation of the type that we have studied is exhibited by insects in which carbohydrate is only a minor source of muscle fuel (moths, grasshoppers). Little is known about muscle metabolism in *Blaberus discoidalis*, although the cockroach, *Periplaneta americana*, does use carbohydrate (Polacek & Kubista, 1960). Among the classic users of carbohydrate for flight energy, the Diptera, activation of trehalase could not be demonstrated. By analysis of substrate concentrations after very short times of flight by blowflies, Sacktor & Wormser-Shavit (1966) have recently shown that hydrolysis of trehalose is a regulated metabolic step in their flight muscle, but the control mechanism is at present unknown. In our preliminary experiments with adenine nucleotides, glucose and several other compounds as possible metabolic regulators of cockroach trehalase, effects were absent or too small to have physiological significance.

It occurred to us that microsomal trehalase might become activated by melting of membrane lipids as a result of temperature rise during muscle activity. The Arrhenius plots for the enzyme from *B. discoidalis*, however, which were of typical form and identical for untreated and activated enzyme, gave no support to this notion.

The total level of trehalase in *Blaberus* muscle is surprisingly high: when fully activated the level relative to total muscle protein is comparable to that demonstrable in flies. The biological reason for the presence of so much masked trehalase in cockroach muscle is not at all clear.

We are grateful to Miss Mary Anne Planeta for skillful help with some of the experiments. This investigation was supported by grants from the Whitehall Foundation and the National Institutes of Health, U.S. Public Health Service (AI-01028).

REFERENCES

Andrews P. (1964). *Biochem. J.* **91**, 222.
Condrea E. & de Vries A. (1964). *Biochim. Biophys. Acta* **84**, 60.
Ehrhardt P. & Voss G. (1962). *J. Insect Physiol.* **8**, 165.
Friedman S. (1960). *Arch. Biochem. Biophys.* **87**, 252.
Garen A. & Levinthal C. (1960). *Biochim. Biophys. Acta* **38**, 470.
Gibson K.O. (1953). *Biochim. Biophys. Acta* **10**, 221.
Gornall A.G., Bardawill C.J. & David M.M. (1949). *J. Biol. Chem.* **177**, 751.
Gussin A.E.S. & Wyatt G.R. (1964). *Amer. Zoologist* **4**, 383.
Gussin A.E.S. & Wyatt G.R. (1965). *Arch. Biochem. Biophys.* **112**, 626.
Hansen K. (1966). *Biochem. Z.* **344**, 15.
Kalf G.F. & Rieder S.V. (1958). *J. Biol. Chem.* **230**, 691.
Lovelock J.E. (1954). *Biochem. J.* **56**, 265.
Lowry O.H. (1957). In *Methods in Enzymology* (S.P. Colowick & N.O. Kaplan, eds.), vol. 4, p. 371. Academic Press, New York.

Lowry O.H., Rosebrough N.J., Farr A.L. & Randall R.J. (1951). *J. Biol. Chem.* **193**, 265.
Marinetti G.V. (1965). *Biochim. Biophys. Acta* **98**, 554.
Martin R.G. & Ames B.N. (1961). *J. Biol. Chem.* **236**, 1372.
Polacek I. & Kubista V. (1960). *Physiol. Bohemoslov.* **9**, 228.
Redman C.M., Siekevitz P. & Palade G.E. (1966). *J. Biol. Chem.* **241**, 1150.
Sacktor B. & Wormser-Shavit E. (1966). *J. Biol. Chem.* **241**, 624.
Saito S. (1960). *J. Biochem.* (*Tokyo*) **48**, 101.
Sawant P.L., Desai I.D. & Tappel A.L. (1964). *Arch. Biochem. Biophys.* **105**, 247.
Siegel L.M. & Monty K.J. (1966). *Biochim. Biophys. Acta* **112**, 346.
Siekevitz P. (1963). *Ann. Rev. Physiol.* **25**, 15.
Slotta K.H. (1960). In *The Enzymes* (P.D. Boyer, H. Lardy & K. Myrbäck, eds.), vol. 4, p. 551. Academic Press, New York, 2nd edition.
Smith D.S. (1962). *Rev. Canad. Biol.* **21**, 279.
Stevenson E.I. & Wyatt G.R. (1962). *Arch. Biochem. Biophys.* **99**, 65.
Wyatt G.R. (1961). *Ann. Rev. Entomol.* **6**, 75.
Wyatt G.R., Gussin A.E. & Gilby A.R. (1965). *Fed. Proc.* **24**, 220.
Wyatt G.R., Kropf R.B. & Carey F.G. (1963). *J. Insect Physiol.* **9**, 137.
Zebe E.C. & McShan W.H. (1959). *J. Cell. Comp. Physiol.* **53**, 21.

TREHALAZY KARALUCHA *BLABERUS DISCOIDALIS*: AKTYWACJA, ROZPUSZCZALNOŚĆ I WŁASNOŚCI ENZYMU Z MIĘŚNIA ORAZ PEWNE WŁASNOŚCI ENZYMU Z JELITA

Streszczenie

1. Mięsień tułowiowy różnych owadów zawiera enzym hydrolizujący trehalozę. U kilku karaluchów i u pasikonika aktywność enzymatyczną homogenatów z mięśnia można, jak wykazano już poprzednio dla motyla *Cecropia*, podwyższyć kilkakrotnie przez kolejne zamrażanie i rozmrażanie homogenatów, natomiast metoda ta nie miała wpływu na aktywność trehalazy mięśniowej much.

2. Aktywność trehalazy u karalucha *Blaberus discoidalis* zlokalizowana jest we frakcji mikrosomalnej otrzymanej wirowaniem przy 105 000 *g*. Aktywność tych preparatów jest specyficzna dla trehalozy; jest ona silnie hamowana przez sacharozę, słabo przez glukozę i D-2-glukozaminę.

3. Aktywność trehalazy w mikrosomalnych preparatach z mięśnia *B. discoidalis* można zwiększyć działaniem detergentów anionowych, kationowych i niejonowych, inkubacją z jadem węża, jak również zamrażaniem i rozmrażaniem. Jad węża surowy lub zagotowany powoduje największą aktywację enzymu (do 10 razy).

4. Aktywowany enzym wykazuje maksymalną aktywność przy pH 6,0. Dla enzymu nieaktywowanego wartości tej nie można było dokładnie oznaczyć, ale wydaje się, że jest ona taka sama. Stała Michaelisa (K_m) dla trehalozy wynosi 3,3 mM dla enzymu nieaktywowanego i 1,7 mM dla enzymu aktywowanego.

5. Z detergentów jedynie dezoksycholan rozpuszcza mikrosomalną trehalazę, ale po jego usunięciu następuje agregacja enzymu. Trehalaza otrzymana w rozpuszczalnej formie pod wpływem jadu węża daje pojedyncze piki zarówno przy rozdziale na kolumnach z Sephadexu G-200 jak i przy wirowaniu w gradiencie sacharozy. Ciężary cząsteczkowe trehalazy otrzymane tymi metodami wynoszą odpowiednio 80 000 i 63 000. Różnica ta może być spowodowana obecnością związanego lipidu.

6. Jelito środkowe *B. discoidalis* zawiera inną trehalazę występującą wyłącznie we frakcji rozpuszczalnej homogenatów. Optimum pH tego enzymu wynosi około 5 a stała Michaelisa (K_m) dla trehalozy 0,5 mM.

Received 28 May, 1966

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

TERESA LASKOWSKA-KLITA and IRENA MOCHNACKA

THE OCCURRENCE OF THE INACTIVE FORM OF *p*-HYDROXYPHENYLPYRUVATE HYDROXYLASE IN THE FROG

Department of Physiological Chemistry, Medical School, Warszawa

1. No *p*-hydroxyphenylpyruvate hydroxylase (EC 1.14.2.2) activity was found in the eggs, tadpole and adult frog. 2. In frog liver and in the tadpole, but not in the eggs, the enzyme activity was revealed after digestion by trypsin or after autolysis. 3. The inhibitor was isolated and some of its properties were determined.

In our Department Michałek-Moricca (1965) in her study on the enzymes of oxidative breakdown of tyrosine in various animals, found tyrosine aminotransferase and homogentisate oxygenase activities in the adult frog *Rana esculenta*, but did not find the activity of the intermediate enzyme, *p*-hydroxyphenylpyruvate hydroxylase which catalyses the formation of homogentisate.

The absence of this enzyme in the frog seemed to be analogous to tyrosinosis, a rare inborn error of metabolism described by Medes (1932) in man. The aim of the present work was to test whether or not *p*-hydroxyphenylpyruvate hydroxylase is missing in the frog at all stages of development, and to gain some information about the causes of its absence.

MATERIALS AND METHODS

Reagents. α,α'-Dipyridyl and 2,6-dichlorophenol indophenol (Biuro Obrotu Odczynnikami Chemicznymi, Gliwice, Poland); tyrosine and L-ascorbic acid (Zakłady Farmaceutyczne, Warszawa, Poland); reduced glutathione and crystalline trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); before use, trypsin was activated according to Baines *et al.* (1964). *p*-Hydroxyphenylpyruvic acid and *p*-hydroxyphenylacetic acid (Koch-Light Laboratories Ltd., Colnbrook, Bucks, England); homogentisic acid, and carboxypeptidase, dialysed and crystallized (Sigma Chemical Co., St. Louis, Mo., U.S.A.); Sephadex G-50 (Pharmacia, Uppsala, Sweden). Crystallized chymotrypsin was a gift from Doc. Dr. P. Szafrański from the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warszawa.

Animals. The experiments were carried out on the frog, *Rana esculenta*, using eggs before and after fertilization, tadpoles, and young and adult frogs. Non-fertilized eggs were obtained directly from the abdominal cavity of females during the mating season; fertilized eggs were collected from spawn. Tadpoles hatched from fertilized eggs were grown at room temperature in tap water changed every 24 hr.; the tank contained also some sand and water plants. The tadpoles were fed dried ground *Daphnia*. Adult frogs were either collected in summer and used immediately or collected late in autumn and kept at 4°.

Enzymic extracts. These were obtained by homogenization of the material in a Potter glass homogenizer with 3 vol. of 0.2 M-K-Na-phosphate buffer, pH 6.5, and centrifugation of the homogenate for 10 min. at 10 000 *g* and 0°. The eggs before homogenization were freed of the jelly-like covering. Whole tadpoles and young frogs were homogenized; from adult frogs only the liver was used. The extracts were prepared just before the experiments.

Protein assay. The tannin micromethod of Mejbaum-Katzenellenbogen (1955) was used, the extinction at 615 mμ being measured in a Bausch & Lomb Spectronic 20 photocolorimeter.

Determination of p-*hydroxyphenylpyruvate hydroxylase.* The enzyme activity was determined by the decrease of the substrate added to the incubation mixture. The composition of the medium and time of incubation were the same as in the experiments of La Du & Zannoni (1955). In 0.1 M-sodium-potassium phosphate buffer, pH 6.5, were dissolved: 2 μmoles of α,α′-dipyridyl, 20 μmoles of reduced glutathione, 25 μg. of 2,6-dichlorophenol indophenol, 5 μmoles of *p*-hydroxyphenylpyruvic acid (freshly dissolved) and 0.5 - 1 ml. of the enzymic extract. The final volume was adjusted to 2.6 ml. with phosphate buffer, pH 6.5. At zero time and after 45 min. incubation at 37°, to samples of 1.3 ml. was added 0.5 ml. of 20% metaphosphoric acid. The precipitated protein was centrifuged off and the content of *p*-hydroxyphenylpyruvate was determined by the enol borate - tautomerase method of Lin, Pitt, Civen & Knox (1958). To 0.5 ml. of the deproteinized solution, 3 ml. of 1 M-sodium borate in 2 M-sodium arsenate, pH 6.5, was added and after 15 min. the extinction at 310 mμ was read against a blank sample, which consisted of 0.5 ml. of the deproteinized solution and 3 ml. of 2 M-sodium arsenate. The content of *p*-hydroxyphenylpyruvate was calculated using the molar extinction coefficient of *p*-hydroxypyruvate - borate complex, 12 000.

Parallelly, the reaction product, homogentisate, was identified by chromatography. To prevent tailing on the chromatogram due to the presence of metaphosphoric acid, samples taken before and after incubation were boiled and kept for 10 min. on a boiling water bath. The precipitated protein was centrifuged off, and 0.2 ml. of the supernatant was applied dropwise on Whatman no. 1 paper and developed in water-saturated *n*-butanol in an atmosphere of formic acid, by the ascending technique, for 16 hr. After drying, the spots were located by spraying with ammoniacal silver nitrate (0.05 M).

Administration of tyrosine and p-*hydroxyphenylpyruvate.* Adult frogs were given tyrosine by intraperitoneal injection, as a suspension in physiological saline solution,

in a dose of 0.33 m-mole per 100 g. body weight; or *p*-hydroxyphenylpyruvate, 0.33 m-mole, with vitamin C (3.6 mg. per 100 g. body wt.) dissolved in physiological saline solution. Vitamin C was applied to prevent the inactivation of the enzyme, which is apt to occur in the presence of excess substrate (Knox & Le May-Knox, 1951). To control animals, a corresponding volume of 0.9% NaCl solution was administered. The urine was collected over a period of 5 or 10 hr. after the injection and phenol compounds were analysed. At different time intervals after the injection, the frogs were killed and *p*-hydroxyphenylpyruvate hydroxylase activity was determined in liver extracts.

Analysis of frog urine. The urine was collected from the cloaca with a micropipette over a determined period at 30 min. intervals. The urines collected from several animals were pooled and phenol compounds were isolated according to Armstrong *et al.* (1956). The urine, usually about 20 ml., was cooled, adjusted to pH 1 - 2 with conc. hydrochloric acid, saturated with sodium chloride (26 g./100 ml.) and extracted three times with an equal volume of ethyl acetate. After evaporation under reduced pressure at 45°, the dry residue was dissolved in anhydrous ethanol and submitted to chromatography by the ascending technique on Whatman no. 4 paper in the solvent system of *n*-butanol - acetic acid - water (4 : 1 : 5, by vol.). The spots were located after 16 hr. runs by spraying with 0.5% solution of diazotized suphanilic acid in 10% Na_2CO_3. The area corresponding to *p*-hydroxyphenylacetate was cut out, eluted with ethyl acetate and rechromatographed on Whatman no. 3 paper in isopropanol - ammonia (20%) - water (8 : 1 : 1, by vol.) and benzene - propionic acid - water (2 : 2 : 1, by vol.). After 20 hr. runs the chromatograms were stained with 0.5% solution of diazotized sulphanilic acid reagent and ammoniacal silver nitrate.

RESULTS

In agreement with the results of Michałek-Moricca (1965), in the liver of frogs during hibernation, in the mating season and in summer no activity of *p*-hydroxyphenylpyruvate hydroxylase could be demonstrated. Moreover, no activity of this enzyme was observed in fertilized and unfertilized eggs, in the tadpoles, or in young frogs just after metamorphosis. The supposition that perhaps a trace activity of the enzyme, undetectable by the methods applied, might exhibit an adaptative increase after intraperitoneal administration of *p*-hydroxyphenylpyruvate, was not confirmed; in the liver of the frogs examined 5 and 10 hr. after the injection, no activity of *p*-hydroxyphenylpyruvate hydroxylase was found.

In the urine of a normal frog, small amounts of *p*-hydroxyphenylpyruvate were found by chromatography, but within 5 - 10 hr. after administration of tyrosine greater amounts were present. After administration of *p*-hydroxyphenylpyruvate, large amounts of this compound were excreted during the first 5 hr., and during the next 5 hr. there appeared another phenolic compound, which was identified as *p*-hydroxyphenylacetate (Fig. 1). At the same time it was found that the standard

p-hydroxyphenylpyruvate, which in *n*-butanol - acetic acid, and benzene - propionic acid solvents gave one spot, in isopropanol - ammonia was resolved into three spots, one of them being *p*-hydroxyphenylacetate.

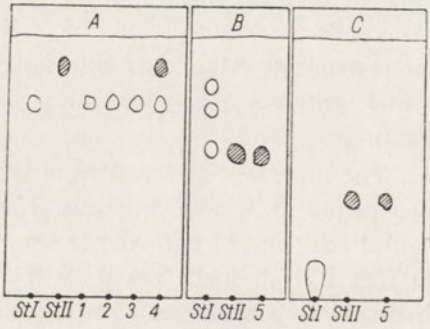


Fig. 1. Chromatographic analysis of phenols isolated from frog urine after administration of tyrosine and *p*-hydroxyphenylpyruvic acid. The phenols were isolated from the urine by the method of Armstrong *et al.* (1956) and chromatographed in the system (*A*): *n*-butanol - water. The area corresponding to *p*-hydroxyphenylacetate was cut out and rechromatographed in (*B*): isopropanol-ammonia, and (*C*): benzene - propionic acid - water. The spots were located with 0.5% solution of diazotized sulphanilic acid in 10% sodium carbonate. *St*, Standards of (*I*), *p*-hydroxyphenylpyruvic acid; (*II*), *p*-hydroxyphenylacetic acid. Phenol compound isolated from: (*1*), normal urine; (*2*), urine collected within 5 - 10 hr. after tyrosine administration; (*3*), urine collected within 5 hr. after *p*-hydroxyphenylpyruvate administration; (*4*), urine collected within 5 - 10 hr. after *p*-hydroxyphenylpyruvate administration; (*5*), rechromatography of the spot of *p*-hydroxyphenylacetate from sample (*4*).

Activation of p-*hydroxyphenylpyruvate hydroxylase*

The absence in frog liver of only one enzyme of the oxidative breakdown of tyrosine suggested the presence of an inhibitor. Michałek-Moricca (1965) found no decrease in *p*-hydroxyphenylpyruvate hydroxylase activity in pike liver after addition of frog liver extract, which demonstrated the absence of a free inhibitor. It was thought possible that the inhibitor might be bound to the enzyme, and therefore the effect of pancreatic enzymes was studied.

In preliminary experiments, livers from several frogs were ground and mixed with 2 volumes of an aqueous extract from ox pancreas, 0.5 mg. of pancreas being taken per 1 g. of liver. The mixture was incubated for 1 hr. at 25°, then cooled to 8°, adjusted with 6 N-acetic acid to pH 5.1 - 5.2, and after 30 min. filtered. The clear solution was adjusted to pH 6.5 and assayed for *p*-hydroxyphenylpyruvate hydroxylase activity. After incubation, homogentisate was found by chromatography. In a non-incubated mixture of frog liver homogenate and pancreas extract no activity was found. On the other hand, incubation of rat liver extract with pancreas did not alter the activity of *p*-hydroxyphenylpyruvate hydroxylase.

Table 1

The effect of digestion on the activity of p-*hydroxyphenylpyruvate hydroxylase in frog liver*

The activity of the liver extract was measured immediately, after 1 hr. autolysis at 25°, and after trypsin and chymotrypsin digestion (100 μg./ml.). Composition of the sample for enzyme assays is given in Materials and Methods. The results are expressed as μmoles of decomposed substrate per 1 g. of liver per 45 min.

Treatment of the extract	Activity	Homogentisate (on chromatography)
None	0.0	—
Autolysis	1.22	+
Chymotrypsin	1.45	+
Trypsin	5.60	+

In further experiments, the effect of trypsin and chymotrypsin (100 μg. per 1 ml. of frog liver extract) as well as autolysis, was studied. After 1 hr. incubation at 25° the hydroxylase activity was determined and in all samples the activity of *p*-hydroxyphenylpyruvate hydroxylase was observed (Table 1). The effect of 1 hr. autolysis was similar to that of chymotrypsin treatment, and the highest activity was obtained after trypsin digestion. Homogentisate was also found in all samples.

Localization of p-*hydroxyphenylpyruvate hydroxylase in liver subcellular fractions*

Ground liver was suspended in an equal volume of 0.25 M-sucrose, homogenized in a Potter glass homogenizer with a polyacryl pestle, then 8 volumes of 0.25 M-sucrose was added, and centrifuged at 0°. The nuclei were obtained by centrifugation at 600 *g* for 15 min. and the mitochondria at 12 000 *g* for 15 min. The nuclei and

Table 2

Localization of p-*hydroxyphenylpyruvate hydroxylase in the subcellular fractions of frog liver*

The activity was determined before and after 1 hr. of digestion by trypsin (100 μg./ml., 25°). For details see text. The activity is expressed as μmoles of decomposed substrate during 45 min.

Fraction	Content of protein in the sample (μg.)	Activity			
		before digestion		after digestion	
		per sample	per 1 mg. protein	per sample	per 1 mg. protein
Nuclei (600 *g*)	20	0	—	0	—
Mitochondria (12 000 *g*)	7.2	0	—	0	—
Post-mitochondrial supernatant	3.2	0.31	0.097	0.58	0.183

mitochondria pellets were washed with 0.25 M-sucrose, suspended in 0.1 M-phosphate buffer, pH 6.5, and homogenized.

In the nuclei and mitochondria (Table 2) no activity of *p*-hydroxyphenylpyruvate hydroxylase was found, either directly or after trypsin digestion. The activity was

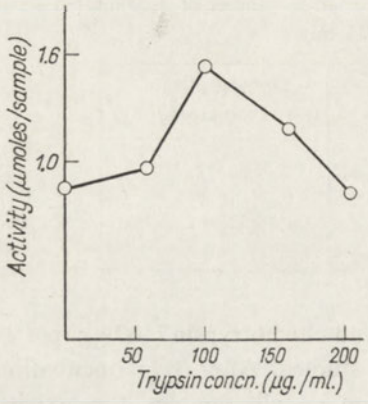


Fig. 2. The effect of trypsin on the activity of *p*-hydroxyphenylpyruvate hydroxylase in the post-mitochondrial supernatant of frog liver cells. The activity is expressed as μmoles of decomposed substrate per sample (0.1 g. liver).

present in the 12 000 *g* supernatant, and it was doubled after trypsin digestion. The activity without trypsin digestion can be explained by autolysis occurring during fractionation.

The optimum amount of trypsin for activation of *p*-hydroxyphenylpyruvate hydroxylase was found to be 100 μg. per 1 ml. of the supernatant which corresponded to 0.1 g. of liver tissue (Fig. 2).

Purification of p-*hydroxyphenylpyruvate hydroxylase*

Post-mitochondrial supernatant was fractionated with ammonium sulphate. The fraction precipitated at 0.4 - 0.7 saturation contained the hydroxylase, the activity of which increased after trypsin digestion. The precipitate was dissolved in 2 - 3 ml. of 0.01 M-phosphate buffer, pH 7.3, and applied to a Sephadex G-50 column. Two fractions were obtained (Fig. 3); fraction *I* had the activity of *p*-hydroxyphenylpyruvate hydroxylase whereas fraction *II* was inactive and, when added to the active enzyme, had an inhibitory effect.

To achieve further purification of the enzyme, fraction *I* was treated with ammonium sulphate and the proteins precipitated at 0.4 - 0.7 saturation were collected, dissolved in 1 - 2 ml. of the above phosphate buffer and passed through a Sephadex G-50 column. Again two fractions were obtained: a major one containing *p*-hydroxyphenylpyruvate hydroxylase activity, and a very small fraction *II* possessing the inhibitor activity.

The applied procedure permitted to obtain a 23-fold purification of the enzyme. The activities at the successive stages of purification before and after trypsin di-

gestion, are presented in Table 3. The fact that no activity before trypsin digestion was detected in the whole extract, but it was found in the 12 000 *g* supernatant and increased after the first fractionation, was due to autolysis occurring during

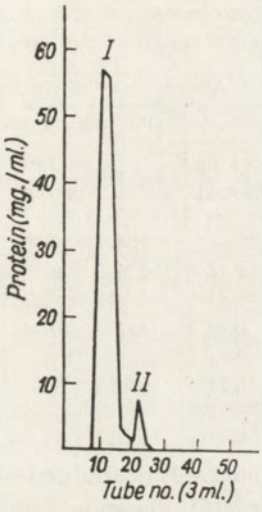


Fig. 3

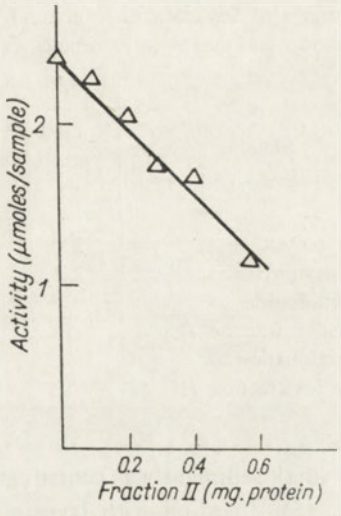


Fig. 4

Fig. 3. Separation on Sephadex G-50 column of the proteins of post-mitochondrial liver supernatant precipitated at 0.4 - 0.7 $(NH_4)_2SO_4$ sat. The Sephadex column (40×1.5 cm.) was equilibrated with 0.01 M-phosphate buffer, the same buffer being used for elution of the protein. Fractions of 3 ml. were collected at 12 min. intervals, and protein was determined.

Fig. 4. The effect of Sephadex fraction *II* on the activity of *p*-hydroxyphenylpyruvate hydroxylase. To the incubation mixture containing 3.2 mg. of protein of Sephadex fraction *I*, were added increasing amounts of fraction *II*.

the applied procedure. The lack of an increase in activity per 1 mg. protein observed after the second fractionation, is probably connected with the removal of cell proteolytic enzymes.

Properties of the inhibitor

The effect of concentration of the inhibitor on *p*-hydroxyphenylpyruvate hydroxylase activity was studied by adding Sephadex fraction *II* to fraction *I*. The degree of inhibition was directly proportional to the amount of the added fraction *II* (Fig. 4). A 50% inhibition was obtained when the weight ratio of fraction *II* to fraction *I* was as 1 : 6.

The activity of the inhibitor was not affected by the following treatments: (1), dialysis for 6 hr. against a 1000-fold volume of 0.01 M-phosphate buffer, pH 7.3, at 0°, with continuous stirring; (2), storage at -20° for two weeks; (3), heating for 30 min. in a boiling water bath; (4), treatment with 2.5% trichloroacetic acid for

Table 3

Purification of p-*hydroxyphenylpyruvate hydroxylase from frog liver*

1st fractionation: the precipitate at 0.4 - 0.7 $(NH_4)_2SO_4$ sat. was submitted to Sephadex G-50 gel filtration. 2nd fractionation: Sephadex fraction I was precipitated at 0.4 - 0.7 $(NH_4)_2SO_4$ sat. and applied again to Sephadex G-50 column. The activity was determined before and after trypsin digestion and expressed as μmoles of substrate decomposed during 45 min. incubation.

Stage	Volume (ml.)	Protein (mg.)	Activity				Degree of purification
			before digestion		after digestion		
			total	per 1 mg. protein	total	per 1 mg. protein	
Whole extract	290	2700	0	—	164	0.06	
12 000 *g* supernatant	223	1190	115	0.10	226	0.19	3.3
1st fractionation (Sephadex fraction *I*)	15	450	105	0.23	152	0.34	5.7
2nd fractionation (Sephadex fraction *I*)	12	41	10	0.24	56	1.6	23

4 hr. (a small sediment was centrifuged off, and the fluid was neutralized with 0.1 N-NaOH); (5), digestion with trypsin at 25° for 1 hr.; (6), digestion with carboxypeptidase for 2 hr. at 37°.

p-*Hydroxyphenylpyruvate hydroxylase in frog ontogenesis*

The hydroxylase activity was examined after trypsin digestion. In the extracts of unfertilized and fertilized eggs, up to the time of hatching of tadpoles, no *p*-hydroxyphenylpyruvate hydroxylase activity could be demonstrated. Tadpoles in the first days after hatching (5 - 11 days of ontogenesis) were not assayed, but on

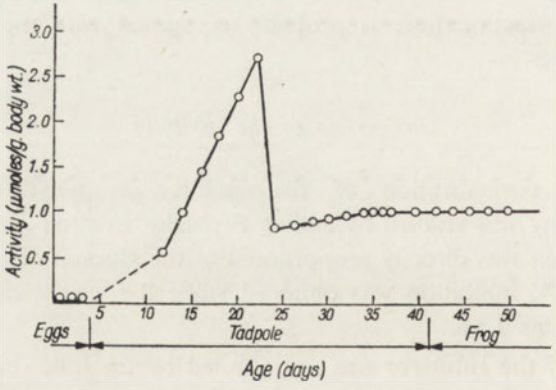


Fig. 5. *p*-Hydroxyphenylpyruvate hydroxylase during development of the frog. The activity was determined in extracts of eggs, whole tadpole and whole young frog, after digestion with trypsin.

the 12th day the enzyme was found (Fig. 5). Its amount per 1 g. body weight increased in the tadpole up to the 22nd day of ontogenesis, then it decreased rapidly reaching on the 25th day the value found for the whole young frog within 12 days after metamorphosis.

DISCUSSION

It is generally known that some proteolytic enzymes occur in the form of inactive proenzymes which may be activated by peptidase digestion. There is, however, little information concerning the occurrence of such pro forms of other enzymes. Szarkowski (1957), who studied tyrosine activity in rye seeds, within a week after harvest found only the catecholase activity; the cresolase activity appeared after trypsin digestion and in seeds stored for over 4 weeks.

In the present work it was found that *p*-hydroxyphenylpyruvate hydroxylase is present in the proenzyme form in the tadpole and adult frog, but not in the eggs. The proenzyme was activated by trypsin digestion or on autolysis of the homogenate. After activation, the inhibitor was separated by Sephadex gel filtration. The obtained compound added to active hydroxylase inhibited its activity. The inhibitor was found to be a non-diffusible compound, soluble in trichloroacetic acid, and it was not destroyed by elevated temperature, and by treatment with trichloroacetic acid, trypsin, and carboxypeptidase.

In the frog, the absence of *p*-hydroxyphenylpyruvate hydroxylase activity, even in the presence of tyrosine aminotransferase and homogentisate oxygenase, apparently excludes the oxidation of tyrosine through the main catabolic pathway. However, the effect of autolysis indicates that the cell possesses an enzyme system capable of converting the proenzyme into the active enzyme. Although in the frog, irrespective of the stage of development and seasonal changes in physiological condition, the presence of active *p*-hydroxyphenylpyruvate hydroxylase could not be demonstrated, it seems possible to assume the existence of a factor stimulating the activation of this enzyme. Tyrosine is a precursor of thyroxine, melanine and other important compounds. The existence of a regulatory mechanism controlling the flow of this substrate through the different metabolic pathways, could be expected.

REFERENCES

Armstrong M.D., Shaw K.N.F. & Wall P.K. (1956). *J. Biol. Chem.* **218**, 293.
Baines N.J., Baird J.B. & Elmore D.T. (1964). *Biochem. J.* **90**, 470.
Knox W.E. & Le May-Knox M. (1951). *Biochem. J.* **49**, 686.
La Du B.N. & Zannoni V.G. (1955). *J. Biol. Chem.* **217**, 777.
Lin E.C.C., Pitt B.M., Civen M. & Knox W.E. (1958). *J. Biol. Chem.* **233**, 668.
Medes G. (1932). *Biochem. J.* **26**, 917.
Mejbaum-Katzenellenbogen W. (1955). *Acta Biochim. Polon.* **2**, 279.
Michałek-Moricca H. (1965). *Acta Biochim. Polon.* **12**, 167.
Szarkowski J. (1957). *Acta Biochim. Polon.* **4**, 129.

WYSTĘPOWANIE NIECZYNNEJ POSTACI HYDROKSYLAZY *p*-HYDROKSYFENYLOPIROGRONIANU U ŻABY

Streszczenie

1. W jajach, kijankach i żabach dorosłych nie wykazano aktywności hydroksylazy *p*-hydroksyfenylopirogronianu.

2. Aktywność pojawiała się po trawieniu trypsyną lub po autolizie homogenatu wątroby żaby i kijanek, ale nie jaj.

3. Wyizolowano inhibitor i oznaczono niektóre jego właściwości.

Received 28 May, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

Z. SZOT and J. GEISLER

INFLUENCE OF FLUORIDE ION ON THE CALCIFICATION PROCESS IN RAT BONE

Department of Radiobiology and Health Protection, Institute of Nuclear Research, Świerk

1. A 10% enhancement of Ca deposition was found in the bone only after oral administration of high doses of NaF. 2. Ca deposition in *in vitro* experiments increased with increasing NaF concentration in the medium. 3. The presence of NaF in the incubation medium decreased the mobilization of calcium from the bone. 4. The living bone, in contrast to the dead bone, had the ability to resist excessive calcification.

Fluorine may be found in small amounts, about 0.5 part per million (p.p.m.), in the blood and soft tissues. In the mineralizing tissues like teeth and bones the amount of fluorine is about 100 or more times higher (Singh, Jolly, Bansal & Mathew 1963). Differences in the content of fluorine in human body are correlated with the concentration of fluorine in drinking water which varies from zero to a few p.p.m. (Jackson & Weideman, 1958; Hodge, 1961). Small amounts of fluorine incorporated into the mineral part of bone or tooth improve its crystallinity, diminish its solubility and reduce resorption processes.

Consumption of water containing more than 2 p.p.m. of fluorine over a period of several years causes mottling of the enamel. Disorders of the bone tissue occur when for a long period of time large doses are incorporated. This can take place in an area where the drinking water is rich in fluorine and in persons who are professionally exposed to this element. Chronic fluorosis is manifested mainly by osteosclerosis, hypercalcification, bone exostoses and calcification of ligaments (Roholm, 1936; Profitt & Ackerman 1964; Zipkin, Shraer, Shraer & Lee, 1963).

Although during the last 20 years the effect of fluorine on bone calcification has been extensively studied, we are still far from an exact knowledge of the mechanism of fluorine action. The aim of the present work was to study the effect of sodium fluoride on the calcification process in rat bone *in vivo* as well as *in vitro*.

MATERIALS AND METHODS

Animals. Wistar albino rats 3 - 6 weeks old, and adult rats weighing 180 - 300 g. were used. The animals, about 3 weeks old, were purchased from the same breeder and kept on standard diet.

Fluorine administration. Rats selected for the experiment were kept on the standard diet except that for 6 - 30 days they received drinking water which contained 10 - 20 mg% NaF. The daily intake of the NaF solution was recorded. Control animals were kept under the same conditions, but were given pure drinking water.

Preparation of bone for examination. The animals were killed by bleeding under ether anaesthesia. The femurs were dissected and cleaned from the adjacent tissue and bone marrow. To determine the effect of fluoride *in vivo*, the bone samples were used for calcium and phosphate assays without further treatment. To determine the effect of fluoride *in vitro*, the femurs from either NaF-treated or untreated animals were cut longitudinally into halves which were both incubated for several hours in a mineralizing solution, with or without the addition of fluoride.

Mineralizing solution. The stock solutions for incubation were prepared according to Fleish & Neuman (1961). One litre of solution contained: (A), 1.4566 g. K_2HPO_4, 8.7227 g. KCl, 1.8419 g. diethylbarbituric acid; (B), 1.2269 g. $CaCl_2 \cdot 6H_2O$, 10.2879 g. KCl, 1.8419 g. diethylbarbituric acid; (C), 11.5552 g. KCl, 1.8419 g. diethylbarbituric acid. The pH values were adjusted to 7.4 with 1 N-KOH.

The stock solutions A, B and C were mixed in a proportion of 7.5 : 12 : 30.5, respectively, then glucose (1 mg./ml.) and $^{45}CaCl_2$ (800 - 1000 disintegrations/min.) were added. The final solution contained 1.605 mM-PO_4^{3-} and 1.344 mM-Ca^{2+}. The "calcium phosphate concentration product" (Ca×P) (Fleish & Neuman, 1961) was therefore equal to 2.16 $(mM)^2$ and calcium : phosphate molar ratio was 0.84. Under these conditions the ionic strength was 0.165 and the pH 7.4.

Bone mineralization in vitro. Samples of a few milligrams of bone were incubated in 50 ml. of the above solution in a water bath at 37° for 5 or 17 - 18 hr. and gently stirred, then calcium and phosphate were determined. The Ca : P ratio in the mineralized bone is one of the main tests which enables a comparison of *in vitro* and *in vivo* systems (Bourne, 1956). In our experiments *in vitro* the Ca : P ratio was 2.06 ± 0.15.

To assess the viability of the bone studied, respiration and glycolysis (Umbreit, Burris & Stauffer, 1951; Barker & Summerson, 1941) were examined after several hours of incubation (Figs. 1 and 2).

Calcium determination. In experiments with oral administration of NaF, the cleaned femurs were cut into small pieces and put for 24 hr. into a 2.5% HCl solution. After centrifugation, the calcium content in the supernatant was estimated manganometrically by titration of calcium oxalate precipitated at pH 8.5. The accuracy of estimation was about 2% in the samples containing 8 - 12 mg% Ca.

In *in vitro* experiments, the increase in calcium content in the bone samples during incubation in the mineralizing solution was determined by the isotopic method. After incubation the samples were rinsed with distilled water and decalcified in 5 ml. of 2.5% HCl for 24 hr. The HCl solution containing the total calcium of the sample was transferred into a special tube (Fig. 3). After the addition of $CaCl_2$ as carrier, calcium ion was precipitated with oxalate on a dish of aluminium foil, 7 mg. per $cm.^2$, at weakly alkaline pH. The centrifuged and dried precipitate

was measured for radioactivity in EKCO scintillation counter type N 664 A. As detector was used a NE 102 scintillating plastic disk 20 mm. in diameter and 0.4 mm. thick (Jaworowski, 1963). The dish with the precipitate was put directly on the scintillating disk and counted for a time sufficiently long to reduce the error of measurement to $\pm$ 5%. The results were corrected for self-absorption of β particles of the sample. In the same way calcium oxalate was precipitated in a known volume of the standard incubation solution and its radioactivity was measured. The amount

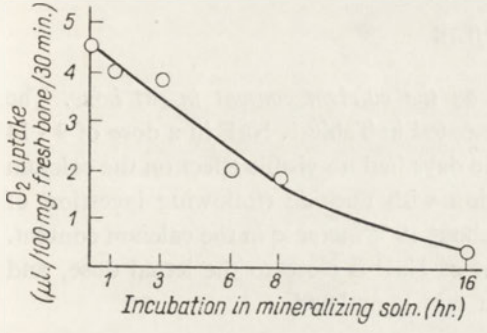


Fig. 1

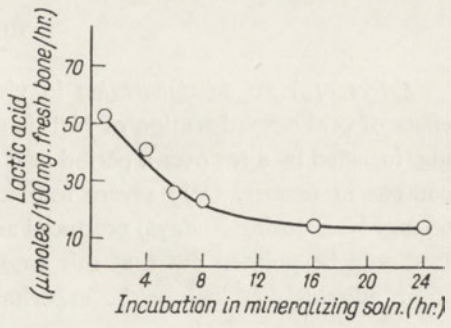
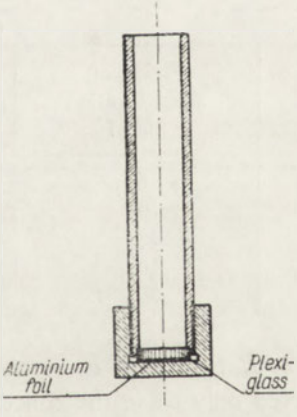


Fig. 2

Fig. 1. Respiration of young rat bone examined after incubation in mineralizing solution.
Fig. 2. Glycolysis in rat bone examined after incubation in mineralizing solution.

Fig. 3. Tube for precipitation of radioactive Ca on the counting dish.



of deposited calcium per mg. of fresh tissue in the proper and control samples can be calculated if the radioactivities and weights of the samples and the ^{40}Ca : ^{45}Ca ratio of the standard solution are known.

The application of ^{45}Ca for determination of the increase of calcification was admissible due to the great excess of the Ca carrier and because the second half of the same bone was used as control. Determination of calcium content by the

chemical and isotopic methods gave very similar results; for 10 samples assayed after incubation the differences between the determinations by the two methods amounted to about 3%.

Determination of phosphate. The bone was dissolved in 2.5% HCl solution, and phosphate content was determined according to the method described by Kemula & Wolfram (1958).

Reagents. AR grade reagents were supplied by Biuro Obrotu Odczynnikami Chemicznymi, Gliwice, Poland. $^{45}CaCl_2$ was produced in U.S.S.R.

RESULTS

Effect of NaF *administration* in vivo *on the calcium content in rat bone.* The effect of oral administration of NaF is presented in Table 1. NaF in a dose of 9 - 14 mg. ingested by a rat over a period of 6 - 18 days had no visible effect on the calcium content in femurs. Only severe intoxication with fluoride (following ingestion of 65 mg. NaF during 30 days) produced an about 10% increase in the calcium content. It should be pointed out that this amount of NaF is close to the lethal dose, and some animals died before the experiment was completed.

Table 1

Effect of oral administration of NaF *on calcium content in rat femur*

Rats 3 - 6 weeks old (weighing 30 - 70 g.) or adult rats (180 - 300 g.) were used. In the bone, calcium was determined by the manganometric method. Mean values ±S.D. are given. 1 mg. NaF = = 0.45 mg. F.

Total dose of NaF (mg.)	Period of administration (days)	Numbers of rats	Calcium in fresh bone (%)	
			Fluorized rats	Control rats
9	6	10	7.85±0.71	8.15±1.00
11	10	5	9.16±1.32	9.30±1.21
12.5	18	7	10.39±0.88	9.92±0.70
14	10	9	14.21±1.12	13.89±0.94
65	30	4	12.14±0.18	10.90±0.53

Effect of NaF in vitro *on calcium deposition in the bone.* The effect of fluoride was observed only when the concentration of F^- ion in the mineralizing solution amounted to 0.3 p.p.m. or more, and calcium deposition increased with increasing fluoride concentration (Table 2). The significance of the differences between the NaF-treated and control parts of the bone was tested by Student's *t* test for paired differences. At 0.1 and 0.2 p.p.m. F^- ion concentration the differences were not significant, but the results obtained in the presence of 0.3 or more p.p.m. of F^- differed from those for the controls by a statistically significant value at 0.02 and 0.01 confidence level, respectively.

Table 2

Effect of fluoride concentration in the incubation medium on calcium deposition in bone

Femurs were taken from 3-4-week-old rats kept on standard diet. The bone was incubated in the mineralizing solution, one half without NaF (control), another with the addition of NaF. Calcium was determined by the isotopic method.

F^- ion concn. (p.p.m.)	Ca deposition (mg./100 g. fresh bone)		Increase in Ca deposition	
	NaF added	Control	(mg./100 g.)	(%)
0.1	1058	1013	45	—0.1
	824	820	4	
	800	830	—30	
	1258	1281	—23	
0.2	565	469	96	2
	571	598	—27	
	331	382	—51	
	573	546	27	
0.3	1900	1610	290	17
	2510	2230	280	
	1920	1830	90	
	2190	1650	540	
0.4	1042	806	236	25
	1008	840	168	
	726	609	117	
	554	428	126	
	533	403	130	
	584	494	90	
1.1	584	455	119	35
	600	391	209	
	569	368	201	
	419	329	90	
	482	416	66	
1.8	2087	1406	681	50
	2030	1437	593	
	1558	956	602	
	1904	1252	652	
	1254	788	466	
	1479	1152	327	
	1189	838	351	
	1446	881	565	

Similar experiments were carried out, but at varying calcium and phosphate concentrations. The results presented in Fig. 4 indicate that the effect of F^- ion at 1.8 p.p.m. concentration was the greater, the higher was the Ca^{2+} concentration at constant PO_4^{3-} (Fig. 4A), the higher PO_4^{3-} concentration at constant Ca^{2+} (Fig. 4B) and the greater Ca×P product at constant Ca : P ratio (Fig. 4C). Generally, the enhancement of the deposition of calcium caused by fluoride always increased with increasing concentration of mineralizing salts.

Effect of oral administration of NaF *on calcium deposition in bone* in vitro. The results of the experiment presented in Table 3 show that the administration to the rat of 10 mg. of fluoride during 7 days had no effect on calcium deposition in bone *in vitro*.

Table 3

Comparison of calcium deposition in bones of fluorized and control rats

Rats were given NaF in drinking water for 7 days (total dose about 10 mg.); control animals were given pure water. The femurs were removed and incubated in the mineralizing solution, then calcium was determined by the isotopic method. Individual results and mean values ±S.D. are given.

Ca deposition (μg./100 mg. fresh bone)	
Fluorized rats	Control rats
997	868
801	780
738	948
909	950
Mean: 861 ± 115	886 ± 140

Table 4

Effect of fluoride addition to the incubation medium on calcium mobilization from the bone in vitro

Into the tail vein of rats weighing 30 - 40 g., 0.2 ml. of aqueous $^{45}CaCl_2$ solution (0.7 μc/ml.) was injected. After four days the femurs were removed and incubated in the mineralizing solution with and without 1.8 p.p.m. F^- ion. After 18 hr., radioactivity was determined in the incubation medium and in the bone.

Incubation medium	Bone sample (mg.)	^{45}Ca content (counts/min.)			Ca mobilized (% of the sum)
		in medium	remaining in bone	sum	
Control	38	684	7940	8624	7.9
F^- ion added	41	346	8355	8701	4.0
Control	47	998	9790	10788	9.3
F^- ion added	44	559	8995	9554	5.9
Control	44	572	7105	7677	7.5
F^- ion added	35	192	7135	7327	2.6

Effect of NaF *on the mobilization of calcium from bone* in vitro. Aqueous $^{45}CaCl_2$ solution, 0.2 ml. (0.7 μc/ml.) was injected into the tail vein of rats weighing 30 - 40 g. After four days the animals were killed and their femurs isolated. One half of the bone was incubated in a non-radioactive solution containing 1.8 p.p.m. F^- ion, the second half in the same solution without the addition of NaF. Then radioactivity was determined in the incubation medium and in the solution obtained by dissolving

the bone sample in 2.5% HCl. The results presented in Table 4 indicate that the calcium content in the incubation medium containing NaF was about half that in control solutions, i.e. respectively, on the average 4.2 and 8.2%.

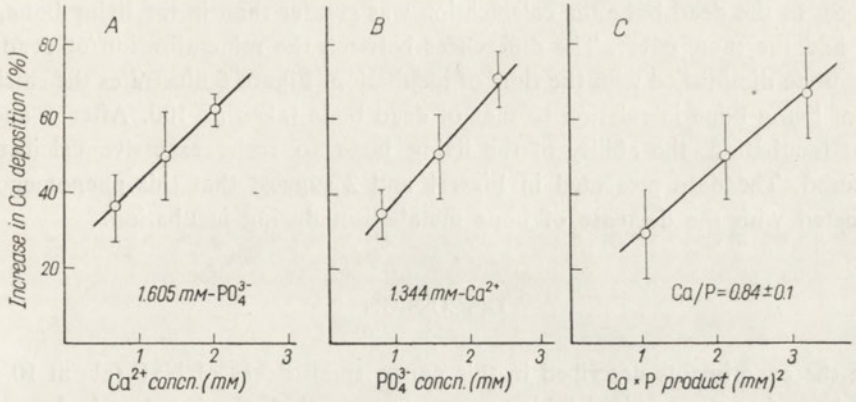


Fig. 4. Effect of F^- ion (1.8 p.p.m.) on calcium deposition in bone under different conditions. Ionic strength 0.165, pH 7.4. *A*, varying concn. of Ca^{2+} and constant PO_4^{3-}; *B*, varying concn. of PO_4^{3-} and constant Ca^{2+}; *C*, varying Ca × P product and constant Ca : P ratio. Calcium was determined by the isotopic method.

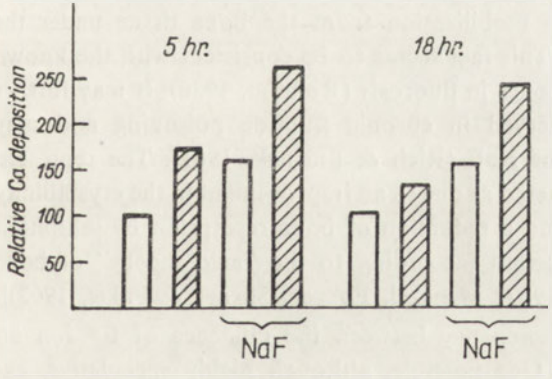


Fig. 5

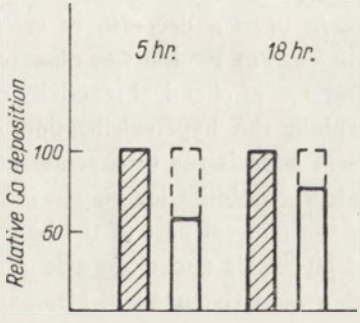


Fig. 6

Fig. 5. Deposition of calcium in living and dead bone incubated in mineralizing solution for 5 and 18 hr., with 1.8 p.p.m. F^- and without NaF. Calcium was determined by the isotopic method. Mean values from 10 experiments are presented. Living bone incubated without NaF was taken as 100. Unshaded columns, living bone; ruled columns, dead bone.

Fig. 6. Resistance against calcification in living bone incubated for 5 and 18 hr. in mineralizing solution. Calcium was determined by the isotopic method. Mean values from 10 experiments are presented. Calcium deposition in dead bone was taken as 100. Unshaded columns, living bone; ruled columns, dead bone.

Effect of NaF *on calcium deposition in living and dead bone* in vitro. Since ethanol does not damage the mineral part of the bone, it was used for devitalization of bone samples. Halves of rat femurs were treated for 20 min. with an excess of 80% ethanol and rinsed with water. The living and dead halves of the bone were incubated for 5 and 18 hr. in the mineralizing solution with and without the addition of NaF (Fig. 5). In the dead bone the calcification was greater than in the living bone, and NaF had the same effect. The differences between the mineralization of dead and living bone diminished with the time of incubation. Figure 6 illustrates the calcification of living bone in relation to that of dead bone taken as 100. After 5 and 18 hr. of incubation, the ability of the living bone to resist excessive calcification decreased. The data presented in Figs. 1 and 2 suggest that this phenomenon is connected with the decrease of bone metabolism during incubation.

DISCUSSION

In the experiments described in this paper, small doses of NaF (about 10 mg.) administered to the rats in drinking water over a period of a few days had no effect on the calcification of the bone. Only after doses as high as 65 mg. NaF administered over a period of 10 days, a 10% increase in bone calcium was observed. In contrast to these results, even very low concentrations of F^- ion gave a remarkable increase in calcium deposition *in vitro* (0.3 p.p.m. F^-, about 17%; 1.8 p.p.m. F^-, 50%).

The *in vitro* deposition of calcium was correlated with the concentration of Ca^{2+} and PO_4^{3-} in the incubation medium. An increase in the concentration of these ions caused a further increase in the effect of F^- ion on calcification.

In vitro a decrease of calcium mobilization from the bone tissue under the influence of F^- ion was observed. This fact seems to be consistent with the known decrease of chemical reactivity of bones in fluorosis (Roholm, 1936). It may further explain the hypercalcification observed in chronic fluoride poisoning and may form a basis for therapeutic use of NaF (Rich & Ensinck, 1961). The repressed calcium mobilization may be interpreted as due to an improvement in the crystallinity of the mineral part of the bone, and a reduction of bone resorption by inhibition of glycolysis and citric acid production [according to the "acid theory" of bone resorption (Neuman & Neuman, 1957; Menczel, Posner, Shraer & Pakis, 1962)].

It is difficult to explain the discrepancy between the influence of F^- ion on calcification *in vitro* and *in vivo*. One possible, although highly speculative, explanation may be that in the young animals used in our experiments the process of bone formation exceeds the bone resorption. In the newly formed osteoid, calcification is so rapid that after a few hours 75% of the total mineralization is attained, but the state of complete mineralization is attained after many months (Johnson, 1964). The relative resistance of growing bone to fluorization *in vivo* is consistent with the experimental finding of Proffit & Ackerman (1964) that DNA synthesis in the bone cells, estimated by uptake of tritiated thymidine and ^{14}C-labelled proline, is not decreased by fluoride in concentrations as high as 10 - 20

p.p.m. We suppose that demonstration of an increase in bone calcium *in vivo* would be possible after blocking of osteoid formation, under conditions of increased bone resorption and also in hypercalcification. To obtain this effect, high doses of NaF would be needed, impossible for administration in experiments *in vivo*.

It was found that the living bone, unlike the dead bone, possesses a resistance against excessive calcification even in solutions containing NaF, which disappears with devitalization. This phenomenon may be explained by the existence of a pH gradient in the skeleton. Nordin's suggestion (cf. MacGregor, 1964) of the presence of a pH gradient between the cell (pH 6.8), the surface of the bone mineral (pH about 7.1) and the extracellular fluid (pH 7.4) is now generally accepted. It may be supposed that the pH gradient is maintained by diffusion due to the limited mobility of the acid end-products of metabolism.

Hydrogen ion concentration is regarded as one of the factors influencing local supersaturation of ions in the tissue fluid which take part in crystal formation. On the other hand, it is known that fluoride inhibits production of lactic acid and reduces concentration of citric acid (Zipkin *et al.*, 1963). It seems therefore reasonable to assume that NaF, by damaging bone cell metabolism, provokes changes in the pH gradient. Local increase in pH may then lead to hydroxy- or fluoroapatite precipitation (Neuman & Weikel, 1954). The controlling effect of fluoride on the calcium deposition postulated by Taves & Neuman (1954) was not observed in our experiments. We ascribe this to the differences in experimental systems used and to differences in the sensitivity of the analytical methods.

We are greatly indebted to Professor Dr. E. Kowalski, in whose Department this work was carried out, for his continued advice and encouragement. Thanks are due to Miss Zofia Długoborska and Miss Irena Ausz for their technical assistance.

REFERENCES

Barker S.B. & Summerson W.H. (1941). *J. Biol. Chem.* **138**, 535.
Bourne G.H. (1956). *The Biochemistry and Physiology of Bone*. Academic Press, New York.
Fleish W. & Neuman W.F. (1961). *Amer. J. Physiol.* **200**, 1296.
Hodge H.C. (1961). *J.A.M.A.* **177**, 313.
Jaworowski Z. (1963). *Nukleonika* **8**, 681.
Johnson L.C. (1964). *Bone Biodynamics* (H.M. Frost, ed.). Little, Brown & Co., Boston, chapter XXIX.
Jackson D. & Weideman S.M. (1958). *J. Pathol. Bacteriol.* **76**, 451.
Kemula W. & Wolfram W. (1958). *Chemia anal.* **3**, 897.
MacGregor J. (1964). In *Bone and Tooth* (H.J.J. Blackwood, ed.) p. 351. Pergamon Press, New York-Oxford.
Menczel J., Posner A.S., Shraer H. & Pakis G. (1962). *Proc. Soc. Exper. Biol. Med.* **110**, 609.
Neuman W.F. & Weikel J.H. (1954). UR-314.

Neuman W.F. & Neuman M.H. (1957). *The Chemical Dynamics of Bone Mineral.* The Univ. of Chicago Press.
Profitt W.R. & Ackerman J.L. (1964). *Science* **145**, 932.
Roholm K. (1936). *Klin. Wochenschrift* **15**, 1425.
Rich C. & Ensinck J. (1961). *Nature* **191**, 184.
Singh A., Jolly S.S., Bansal B.C. & Mathew C.G. (1963). *Medicine* **42**, 229.
Taves D.R. & Neuman W.F. (1964). *Arch. Biochem. Biophys.* **108**, 390.
Umbreit W.W., Burris R. & Stauffer J.F. (1951). *Manometric Techniques and Tissue Metabolism.* Burgess Publishing Co., Minneapolis, Chapter I and VIII.
Zipkin I., Shraer R., Shraer H. & Lee W.A. (1963). *Arch. oral. Biol.* **8**, 119.

WPŁYW JONU FLUORKOWEGO NA PROCES KALCYFIKACJI W KOŚCI SZCZURA

Streszczenie

1. Po podaniu szczurom w wodzie pitnej NaF, wzrost zawartości Ca o 10% w kościach stwierdzono jedynie przy stosowaniu wysokich dawek NaF.
2. *In vitro*, inkubując kości w płynie mineralizującym, stwierdzono, że przyrosty Ca zwiększały się ze wzrostem stężenia NaF.
3. Obecność NaF w środowisku inkubacyjnym zmniejszała mobilizację Ca z kości.
4. Stwierdzono, że kość żywa przeciwdziała nadmiernej kalcyfikacji, natomiast kość martwa zdolności tej nie wykazuje.

Received 28 May, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

S. BURACZEWSKI*, LUCYNA BURACZEWSKA* and J.E. FORD

THE INFLUENCE OF HEATING OF FISH PROTEINS ON THE COURSE OF THEIR DIGESTION

National Institute for Research in Dairying, Shinfield, Reading, England

1. A study was made of the effects of heating on the course of digestion of cod muscle protein. In experiments with rats, the contents of stomachs and small intestines were analysed in relation to the heat treatment of the test protein and the time elapsed since the test meal, and the results compared with those from digestibility tests *in vitro*. 2. Findings *in vivo* and *in vitro* were broadly similar. 3. With increasing severity of heating the solubility of the test protein decreased, as did the rate at which it was hydrolysed by pepsin and subsequently by pancreatin and erepsin. 4. An unexpected finding was a relatively very high concentration of free α-amino nitrogen in the intestinal contents of rats given severely heated protein, and it is postulated that the accumulated intermediate products of protein digestion might hinder the absorption of amino acids by saturating the sites involved in the transport of the amino acids across the mucosal barrier.

The amino acid composition of a food protein, as revealed by classical analysis, may be said to represent its potential nutritive quality, but other characteristics may be of importance in determining its value for the animal. Of these, digestibility and the biological availability of its amino acids are of first importance, perhaps more especially among protein foods that have been heated in manufacture.

It has been shown (cf. Ford, 1962; 1964; 1965; Miller, Carpenter & Milner, 1965) that heat processing may cause little or no change in the amino acid composition of a food protein but may yet greatly impair its nutritive quality. In a series of twelve whale meat meals of broadly similar amino acid composition, Bunyan & Price (1960) found wide variation in nutritive quality for the rat. From the poorer meals, which contained relatively low proportions of available amino acids as judged in chemical and microbiological tests, correspondingly low percentages of the nitrogen absorbed from the gut were retained by the rat.

The question arose (Ford, 1964) whether this finding is wholly explainable in terms of differences in the patterns of amino acids absorbed from the different qualities of meal.

Ford & Salter (1966) subjected portions of freeze-dried cod fillet to different conditions of heat treatment before digesting them *in vitro* with pronase, or

* Present address: The Institute of Animal Physiology and Nutrition, Jabłonna, near Warsaw.

successively with pepsin, pancreatin and erepsin, or with pepsin and papain. The digestions were conducted under "static" conditions in flasks and also in Sephadex gel G-10 in such a manner that the reaction products were continuously removed from the site of action of the digesting enzymes. The different digests were then passed in turn through a calibrated column of Sephadex gel G-25 and each was resolved into three fractions containing "soluble proteins", "peptides" and "free amino acids". The distribution of several amino acids in these different fractions was determined and assessed in relation to their availabilities in the original test proteins as measured in microbiological and chemical tests.

In the study now presented we have investigated with rats the course of digestion *in vivo* of these same cod fillet preparations. The contents of the stomach and small intestine have been analysed in relation to the heat treatment of the test protein and the time elapsed since the test meal, and the results compared with those obtained from digestibility tests *in vitro*.

EXPERIMENTAL

Test proteins. The test proteins were prepared from fillets of cod (*Gadus morrhua*) which were first minced and freeze-dried as described by Ford (1965). Portions of the meal weighing about 250 g. were spread in layers about 1.5 cm. deep in stainless steel trays, which were then heated in an air oven for 18 hr., one portion at a temperature of 135° and the other at 145°.

Tests in vitro. The test preparations of cod fillet were digested *in vitro* successively with pepsin, pancreatin and erepsin as described by Ford & Salter (1966). The enzymic digests were then fractionated in a column of Sephadex gel G-25 that had been calibrated with "markers" of known molecular weight (cf. Ford, 1965). A portion of each fraction was hydrolysed with 4 N-HCl for 3 hr. at 120° and then used for the estimation of α-amino nitrogen, by reaction with the modified ninhydrin reagent of Moore & Stein (1954). A standard response curve was prepared with known graded concentrations of leucine and the amount of material present in each fraction was calculated in terms of "leucine equivalent" by reference to this standard curve. A full account of the procedure is given by Ford (1965) and Ford & Salter (1966).

Tests with rats. Adult male hooded Norwegian rats were used weighing 300 to 400 g. Until required for the tests they were maintained on "Spillers Laboratory Small Animal Diet (autoclaved)" manufactured by Messrs. Spillers Ltd., Technological Research Station, Station Road, Cambridge.

About 40 hr. before being offered the experimental feeds the rats were weighed and placed in tubular anticoprophagy cages of the type described by Metta, Nash & Johnson (1961). The test proteins were given after an 18 hr. fasting period, in amounts calculated to supply 500 mg. protein per 300 g. rat body weight. For some experiments the test proteins were given mixed with an equal weight of sucrose.

The test meals were eaten within 10 - 15 minutes.

Collection of samples. At 1, 2 or 3 hr. after consuming the test meal the rats

were anaesthetized and the gut exposed. The small intestine, from the ileum to the pylorus, was removed and its contents washed into a beaker with 70 - 80 ml. warm 0.9 % NaCl solution. The contents of the stomach were similarly washed into a beaker with 20 - 30 ml. of saline solution.

Each of the three test proteins (unheated cod fillet, cod fillet heated 18 hr. at 135° and 18 hr. at 145°) was given to 6 rats, which were killed and their stomach and intestinal contents collected 2 hr. after the test meal. In addition the unheated cod fillet and the cod fillet heated at 145° were each given with sucrose to 3 groups of 6 rats which were killed at 1, 2 or 3 hr. after the test meal. All the extracts were cooled quickly in an ice bath and prepared for analysis as quickly as possible after collection.

Analysis of the gut contents. The cooled contents of the stomachs and intestines were centrifuged at 1250 *g* for 20 min. and the supernatant liquors decanted. The residues were suspended in water and centrifuged again, and the washings combined with the original supernatant liquor. The residues were dried, weighed and their nitrogen content determined by micro-Kjeldahl analysis.

The supernatant liquors were further diluted with saline, to 50 ml. for the extracts of stomach contents and to 120 ml. for the extracts of intestinal contents. Portions of each were taken for the measurement of dry matter and nitrogen content. The remaining intestinal contents were combined in such a manner that each made an equal contribution to the total soluble nitrogen. Thus, pooled samples were prepared of the soluble intestinal contents of each of the test groups of 6 rats.

These pooled samples were freeze-dried and redissolved in enough 0.02 M-phosphate buffer of pH 7.6 to give about 10 % concentration of NaCl. There was generally a small insoluble residue, which was removed by centrifuging. This contained less than 1 % of the nitrogen and was discarded. The clear supernatant was fractionated in a column of Sephadex gel G-25 under conditions similar to those employed for the *in vitro* enzymic digests. Soluble protein and peptides of molecular weight greater than about 4000 were eluted in the 80 - 100 ml. fractions; peptides of mol. wt. between 4000 and about 250 emerged in five 10 ml. fractions between 100 and 150 ml. and the free amino acids excepting only tryptophan in six fractions between 150 and 210 ml.

A portion of each fraction was taken and assayed for α-amino nitrogen as described above.

RESULTS AND DISCUSSION

The column of Sephadex gel G-25 used for the analysis of our test extracts was calibrated with the following "marker" compounds: cytochrome *c*, from horse heart, mol. wt. 12 400 (Sigma Chemical Co.); insulin B chain, mol. wt. 3483 and bacitracin A, mol. wt. 1470 (both kindly given by Dr. P. Andrews of this Institute), polymixin B sulphate, mol. wt. 1447 (Burroughs Wellcome & Co.); various amino acids (British Drug Houses Ltd.). The general relationship (cf. Andrews, 1964) — elution volume against log(molecular weight) — is only approximate and marked

deviations are sometimes observed especially among substances of lower molecular weight. But for the purposes of the present study it has been assumed that proteins, peptides and amino acids in the test preparations appeared in the column effluent in order of decreasing molecular size, as did the marker compounds.

Digestion in vitro

Figure 1 shows the distribution of α-amino nitrogen in the Sephadex-gel fractions obtained from the test fish meals after digestion in flasks with pepsin, or successively with pepsin, pancreatin and erepsin.

Pepsin digestion of the unheated fish meal yielded predominantly small peptides of mol. wt. in the approximate range 4000 - 200, with a pronounced peak concentration in the region of mol. wt. 1500. The digest contained a small proportion, about 10%, of material of mol. wt. $>$ 4000 which for the purposes of this discussion may be called "undigested dissolved protein", and a similarly small proportion of "free amino acids". After further digestion of the pepsin digest with pancreatin and erepsin the peak concentration of α-amino nitrogen was found as "free amino acids", with some smaller peptides. There was very little residual "undigested dissolved protein" and larger peptides.

Most of the nitrogen in the test proteins was rendered soluble by the successive action of the digesting enzymes. In the digest of the unheated meal 0.9% of the nitrogen remained undissolved, and 1.7% and 5.0% in the digests of the meals heated respectively at 135° and 145°. Nevertheless the dissolved proteins from these heated meals were far from being completely digested. Heating retarded the course of the pepsin digestion, and of the subsequent hydrolysis with pancreatin and erepsin. With increasing severity of heating the free amino acids component of the digests decreased in amount and the peptide and "undigested dissolved protein" increased.

At the same time the free amino acids component became increasingly deficient in several amino acids relative to their content in the unheated meal, and notably in lysine and the sulphur-containing amino acids. Evidence for this marked differential effect of heating in retarding the enzymic release of several amino acids is presented and discussed by Ford & Salter (1966). But for our present purposes we are concerned mainly with the effects of heat damage upon the broader patterns of protein digestion evident in the profiles of the graphs in Fig. 1.

Digestion in vivo

Cod proteins fed alone. Figure 2 shows the distribution of total nitrogen, measured by micro-Kjeldahl analysis, between the stomachs and intestinal contents of rats two hours after being given the different qualities of test protein. There was little difference in the amounts of nitrogen remaining in the stomach. There were however large differences in the nitrogen contents in the intestine. In the rats given the heated proteins the intestine contained a high concentration of undissolved nitrogen,

whereas in the rats fed the unheated protein the level was very low and similar to that in the control fasted rats. The content of dissolved nitrogen was also much higher in the intestinal contents of the rats given the heated proteins than it was in the rats given the unheated meal. This in turn was higher than the content in the fasted rats.

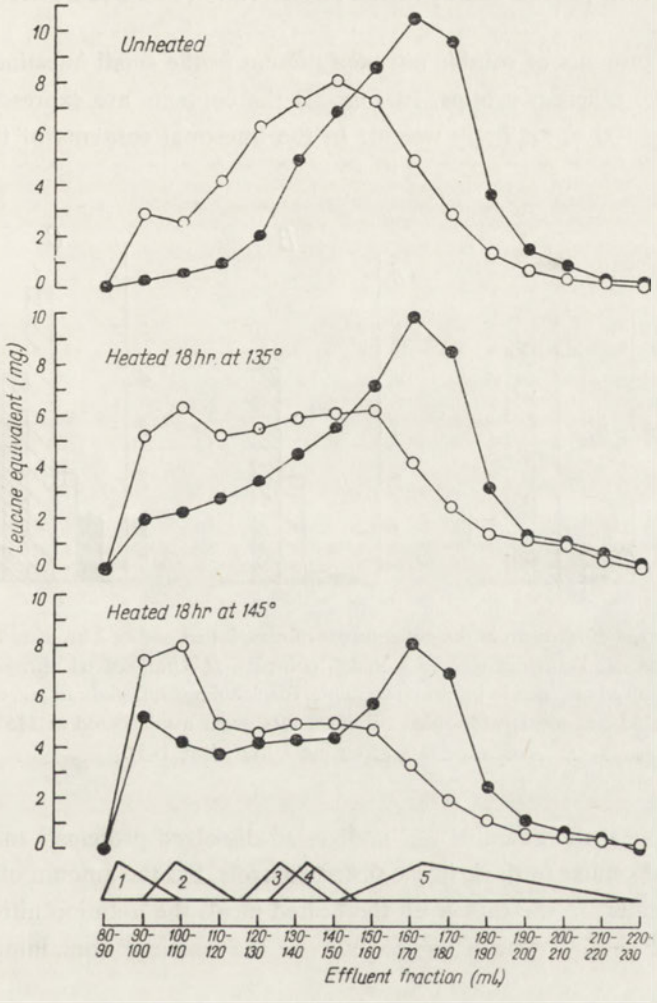


Fig. 1. Fractionation by Sephadex gel G-25 of enzymically digested cod fillet preparations. α-Amino nitrogen content of the fractions (expressed as "leucine-equivalent") was measured by reaction with ninhydrin after hydrolysis of the fractions with acid. The figure shows the distribution of α-amino nitrogen in the test samples containing 16 mg. of nitrogen: (○), digested with pepsin and (●), further digested with pancreatin and erepsin. The numbered triangles at the base of the figure depict the positions of the "markers" in the effluent: *1*, cytochrome *c*, mol. wt. 12 400; *2*, insulin B chain, mol. wt. 3483; *3*, polymixin B sulphate, mol. wt. 1447; *4*, bacitracin A, mol. wt. 1470; *5*, a mixture of 18 amino acids.

Figure 3A shows the distribution of α-amino nitrogen in the "soluble" intestinal contents, expressed per 0.5 mg. of total soluble nitrogen. There was a higher proportion of "undigested dissolved proteins" in the fasted control rats than in the rats given the test proteins. The peptide fraction was markedly higher in the rats given the heated proteins than in the fasted controls and in the rats given the unheated protein. In all four groups the greater part of the total nitrogen was present as free amino acids, in marginally higher concentration in the rats given the unheated protein.

The total amounts of soluble nitrogen present in the small intestine were widely different in the different groups. In Fig. 3B the contents are expressed as leucine equivalent per 100 g. rat body weight. In the intestinal contents of the rats given

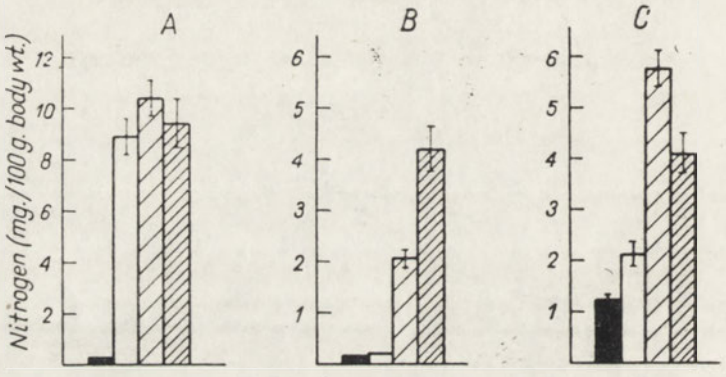


Fig. 2. Distribution of nitrogen in the gut contents of rats, fasted and at 2 hr. after being given cod fillet preparations. *A*, Total nitrogen in stomach contents. *B*, Undissolved nitrogen in intestinal contents. *C*, Dissolved nitrogen in intestinal contents. Black columns, fasted rats; unshaded columns, rats given unheated cod meal; wide-ruled columns, rats given meal heated at 135°; narrow-ruled columns, rats given meal heated at 145°.

the unheated meal the amounts of "undigested dissolved proteins" and "peptides" were small and similar to those in the fasted controls, but the amount of "free amino acids" was greater. In the rats given the heated meals the α-amino nitrogen content of all the fractions was much higher, even in the fractions containing free amino acids.

From Fig. 2 it is apparent that these marked differences in the composition of the intestinal contents did not simply reflect corresponding differences in the rates of stomach emptying and were probably therefore related to differences in the rates of passage in the intestine, and of digestion and absorption. An unexpected finding was that despite these higher intestinal levels of free amino acids present in the rats given the heated meals than in the rats given the unheated meal, the levels of free amino acids in the portal blood were much lower (Buraczewski, 1966).

It seems possible that the presence of a large accumulation of undigested peptides saturates the absorption sites involved in the transport of amino acids across the mucosal barrier, and further work is now in progress to investigate this possibility.

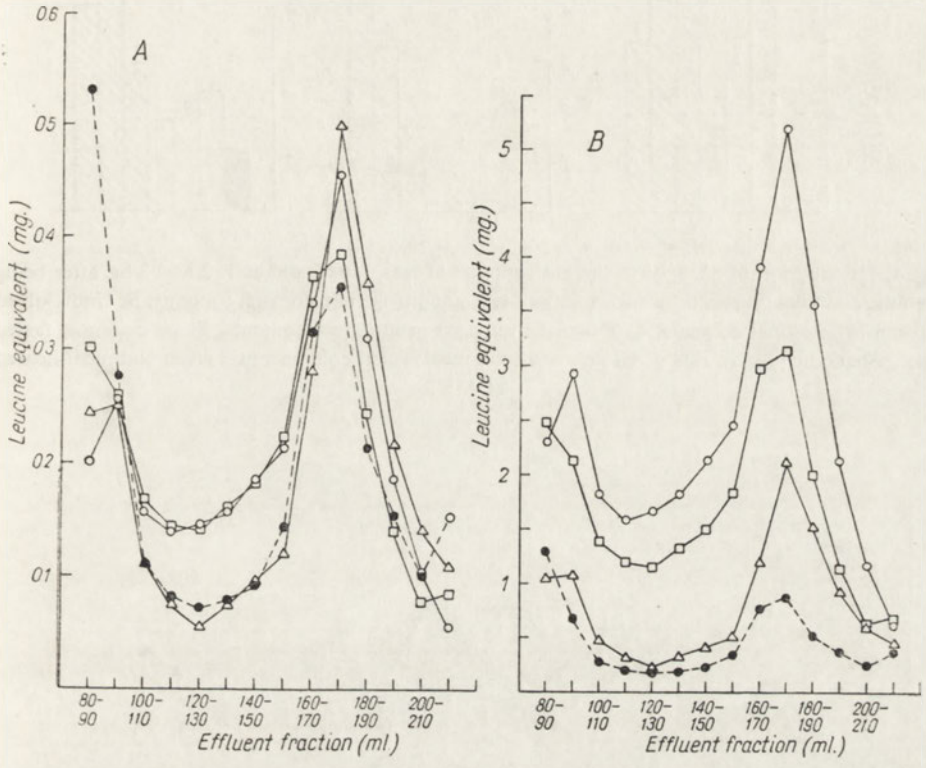


Fig. 3. Fractionation by Sephadex G-25 gel filtration of dissolved intestinal contents of rats, fasted and at 2 hr. after being given cod meals. α-Amino nitrogen was measured by reaction with ninhydrin after hydrolysis of the fractions with acid. Results expressed: *A*, as mg. leucine-equivalent per 0.5 mg. nitrogen; *B*, as mg. leucine-equivalent per 100 g. rat body weight. ●, Values for fasted rats; △, rats given the unheated cod meal; ○, rats given the cod meal heated at 135°; □, rats given the cod meal heated at 145°.

The meal heated at 145° gave a smaller accumulation of α-amino nitrogen than did the meal heated at 135° (Fig. 3B). The explanation for this apparent anomaly is that a higher proportion of the nitrogen in the more severely heated meal remained undissolved and was therefore excluded from the analysis.

Cod proteins fed with sucrose. Figure 4 shows the composition of the nitrogen in the intestine, and the amount of nitrogen remaining in the stomach at 1, 2 and 3 hr. after giving cod meal, unheated and heated at 145°, together with sucrose. In presence of sucrose the nitrogen of the heated protein left the stomach more slowly than did that of the unheated protein. The amounts of undissolved nitrogen in the intestinal contents of rats given the unheated meal were very small, and similar

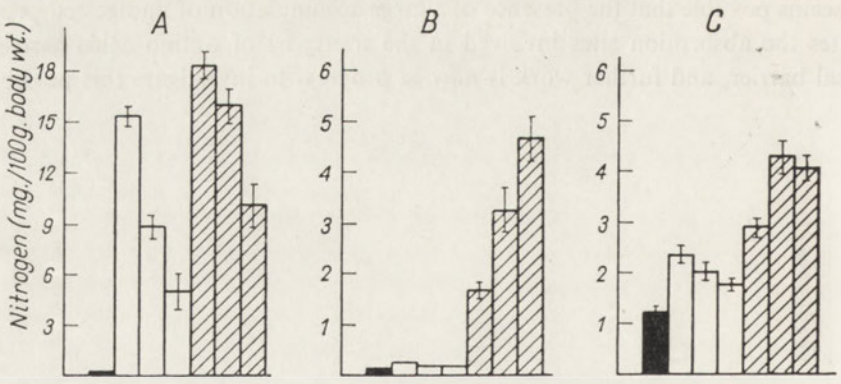


Fig. 4. Distribution of nitrogen in the gut contents of rats, fasted and at 1, 2 and 3 hr. after being given the cod fillet preparations with sucrose. *A*, Total nitrogen in stomach contents. *B*, Undissolved nitrogen in intestinal contents. *C*, Dissolved nitrogen in intestinal contents. Black columns, fasted rats; unshaded columns, rats given unheated cod meal; ruled columns, rats given cod meal heated at 145°.

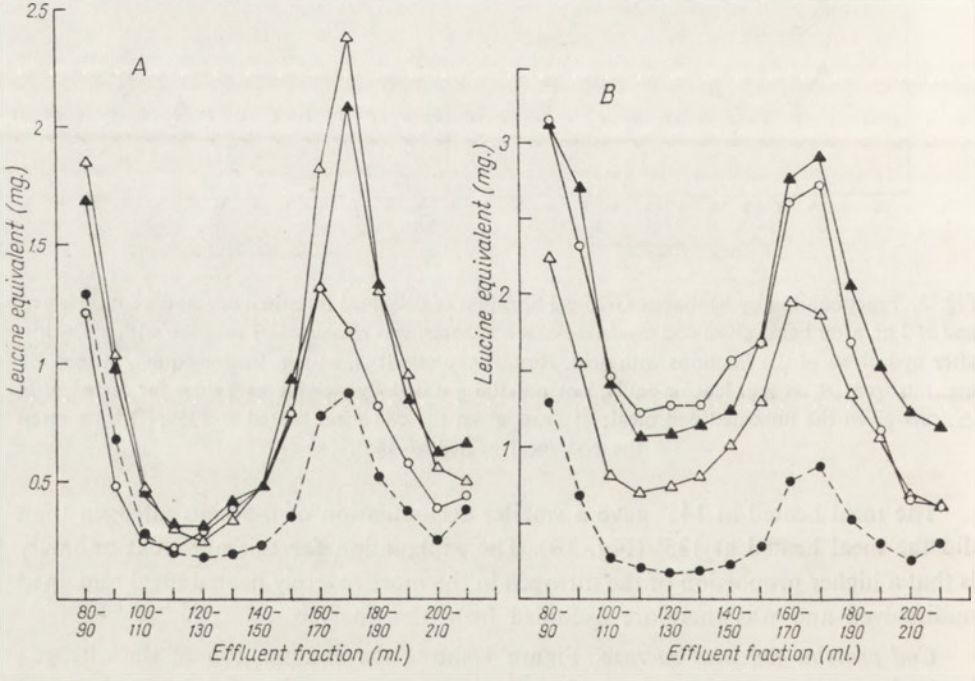


Fig. 5. Fractionation by Sephadex G-25 gel filtration of dissolved intestinal contents of rats, fasted and 1, 2 and 3 hr. after being given cod meals with sucrose. α-Amino nitrogen was measured by reaction with ninhydrin after hydrolysis of the fractions with acid. Results: *A*, for rats given unheated meal and *B*, for rats given meal heated at 145°. ●, Values for fasted rats; △, 1 hr. after feeding; ▲, 2 hr. after feeding; ○, 3 hr. after feeding.

to those in the fasted controls. In the rats given the heated meal there was a large accumulation of undissolved nitrogen in the intestine, which increased with time. Similarly the content of dissolved nitrogen was much higher than in the rats given the unheated protein, as it was when the test proteins were given alone, without sucrose.

The composition of the soluble α-amino nitrogen in the intestinal contents, expressed per unit of nitrogen, was closely similar to that depicted in Fig. 3, and we have thought it unnecessary to reproduce the experimental data here. Again the proportion of peptides was markedly higher, and of free amino acids was marginally lower, in the soluble nitrogen from the intestinal contents of the rats given the heated protein. Thus the administration of sucrose with the test meal caused no change in the composition of the soluble α-amino nitrogen in the intestine. The main effect of the sucrose was apparent in the delayed rate of emptying of the nitrogen from the stomach of the rats given the heated protein.

Figures 5A and B illustrate the changes with time after feeding in the content, per 100 g. rat body weight, of the different soluble α-amino nitrogen fractions in the intestinal contents of rats given the different test proteins together with sucrose.

With increasing time after ingestion of the unheated protein (Fig. 5A) the amounts of undigested protein and free amino acids diminished, whereas the net amount of peptide remained unchanged. In contrast (Fig. 5B) after ingestion of the heated protein the amounts of undigested soluble protein and free amino acids increased to a maximum value at about 2 hr. The amount of peptide increased progressively up to 3 hr. As when the test proteins were fed alone, the amounts of undigested protein, peptide and free amino acids were all generally much greater in the intestinal contents of the animals given the heated protein.

The heated cod meals were much less soluble in the gut than was the unheated meal. It seems clear that in these heated meals the whole process of digestion was retarded with the result that insoluble protein, and soluble undigested proteins and peptides accumulated. It is highly unlikely that these accumulations were caused by any lack of proteolytic enzymes. Indeed, Buraczewski (1966) found that in rats given the heated fish meal the proteolytic activity of the contents of the middle and distal lengths of the small intestine was nearly double that in rats given the unheated meal. The question then arises, to what extent were the higher levels of undigested soluble protein and peptide a measure of an increased secretion of digestive enzymes. An alternative explanation, which we think more likely, is that the presence of the large accumulation of undigested food protein substrate protected the gut enzymes from self-digestion [cf. Twombly-Snook & Meyer (1964)]. What proportion of the accumulation of dissolved nitrogenous material was of endogenous origin is still uncertain.

GENERAL CONCLUSIONS

There was a broad similarity between the results of the tests *in vivo* and *in vitro* and it was evident from both that, with increasing severity of heat treatment, the solubility of the test protein decreased, as did the rate at which it was hydrolysed

by pepsin and subsequently by pancreatin and erepsin. The heated preparations were apparently much less digestible *in vivo* than *in vitro*, but this difference can be partly attributed to the shorter time of exposure to the action of digestive enzymes, 2 hr. as compared with 72 hr. Even so, the prolonged period of digestion employed in these *in vitro* tests liberated considerably more lysine, isoleucine and methionine from the heated protein preparations than was measured in direct feeding experiments with rats (cf. Ford & Salter, 1966) and it seems in fact that digestion *in vivo* of the heat-damaged meals was far from complete, and was comparatively inefficient.

No analysis was made of the amino acid composition of the components of the *in vivo* digests, and so we have as yet no confirmation of the *in vitro* finding (Ford & Salter, 1966) that heating had a marked differential effect in retarding the enzymic release of certain amino acids. However, the results of growth assays with rats, as of the microbiological assays of *in vitro* enzymic digests (Ford & Salter, 1966), showed considerable differences in the extent to which lysine, methionine and isoleucine became biologically unavailable during severe heating, and it is probable that much of the fall in nutritive quality can be attributed to this difference between the rates of enzymic release of different amino acids.

Buraczewski (1966) compared the levels of free amino acids in the portal and systemic blood plasma of rats at two hours after their being given these test proteins, with and without sucrose. In rats given the unheated protein without sucrose the levels of free amino acids were markedly higher in the portal than in the systemic blood plasma. This difference was much smaller in rats given the meal heated at 135°, and was negligible for the meal heated at 145°. Feeding sucrose with the test proteins gave a different picture. For the unheated protein the differences between portal and systemic levels were smaller, and for the heated meals the differences were increased, especially for the meal heated at 135°. Besides this effect on amino acids uptake, sucrose had a pronounced effect in retarding the passage of the heated protein from the stomach. It seems that the course of digestion of a food protein, and the utilization of its amino acids, may be considerably influenced by the dietary context in which it is eaten.

An unexpected finding was the relatively high concentration of free amino nitrogen in the intestinal contents of the rats given the heated fish protein. It is an interesting question whether a large accumulation of intermediate products of protein digestion might hinder the absorption of amino acids by saturation of the absorption sites. Whatever the reason for it, some proportion of the free amino acids in the small intestine may in consequence pass to the large intestine and there enter into the metabolism of the gut microflora. The fate of the residual undigested peptide and protein is as yet unknown, and further investigation is required into the forms in which their nitrogen is excreted.

We thank Dr. J. W. G. Porter for his helpful interest in this work. This work was in part supported by Grant no. AM-06771 from the National Institutes of Health, Bethesda, Maryland, U.S.A.

REFERENCES

Andrews, P. (1964). *Biochem. J.* **91**, 222.
Bunyan, J. & Price, S.A. (1960). *J. Sci. Fd Agric.* **11**, 25.
Buraczewski, S. (1966). *Factors affecting amino acids in the blood.* Doctorate thesis, University of Reading.
Ford, J.E. (1962). *Brit. J. Nutr.* **16**, 409.
Ford, J.E. (1964). *Brit. J. Nutr.* **18**, 449.
Ford, J.E. (1965). *Brit. J. Nutr.* **19**, 277.
Ford, J.E. & Salter, D.N. (1966). *Brit. J. Nutr.* **20**, 843.
Metta, V.C., Nash, L. & Johnson, B.C. (1961). *J. Nutr.* **74**, 473.
Miller, E.L., Carpenter, K.J. & Milner, C.K. (1965). *Brit. J. Nutr.* **19**, 547.
Moore, S. & Stein, W.H. (1954). *J. biol. Chem.* **211**, 907.
Twombly-Snook, J. & Meyer, J.H. (1964). In *The Role of the Gastro-Intestinal Tract in Protein Metabolism* (H.N. Munro, ed.) p. 97. Blackwells, Oxford, England.

WPŁYW OGRZEWANIA BIAŁEK RYBNYCH NA PRZEBIEG ICH TRAWIENIA

Streszczenie

1. Badano zawartość frakcji azotowych w treści pokarmowej żołądka i jelit cienkich szczurów, żywionych nieogrzewaną i ogrzewaną (w temp. 135 i 145°) mączką z mięśni dorsza. Próby treści pobierano w różnych okresach czasu po spożyciu paszy przez zwierzęta.

2. Stwierdzono, że wraz ze wzrostem temperatury ogrzewania malała rozpuszczalność i szybkość hydrolizy białka.

3. Podobne wyniki otrzymano traktując mączki pepsyną, lub kolejno pepsyną, pankreatyną i erypsyną, *in vitro*.

4. Nieoczekiwanie wysoką zawartość wolnego α-aminowego azotu stwierdzono w jelicie cienkim szczurów po spożyciu mączki ogrzewanej w temp. 145°. Przypuszcza się, że nagromadzone pośrednie produkty rozkładu białek utrudniają absorpcję aminokwasów przez wysycenie ośrodków czynnych w transporcie tych związków poprzez błonę śluzową jelita.

Received 27 June, 1966

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

J. BOGUSŁAWSKI, W. WALCZAK and T. KŁOPOTOWSKI

REVERSAL BY SERINE AND POTENTIATION BY GLYCINE OF THE INHIBITORY EFFECT OF 1,2,4-TRIAZOLE ON GROWTH OF *SALMONELLA TYPHIMURIUM*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa 12.

1. 1,2,4-Triazole inhibits transiently the growth of *Salmonella typhimurium*. The inhibition can be relieved by serine, cysteine, homocysteine or methionine. Adenine or glycine increase the inhibition. 2. Presumably, triazole inhibits the generation of one-carbon units from glycine. The enhancement of inhibition by glycine may be due to the feedback inhibition of serine biosynthesis. The similar effect of adenine may result from the repression of the enzymes involved in the metabolism of one-carbon units.

It is now well established that 3-amino-1,2,4-triazole[1] inhibits competitively an enzyme of histidine biosynthesis, imidazoleglycerolphosphate dehydratase (EC 4.2.1.19) (Hilton, Kearney & Ames, 1965; Kłopotowski & Wiater, 1965; Wiater & Kłopotowski, to be published). In addition, 3-AT inhibits a process required for purine synthesis (Hilton, Kearney & Ames, 1965; Kłopotowski & Bagdasarian, 1966). Triazole and AET are also competitive, but weaker, inhibitors of the dehydratase of *Saccharomyces cerevisiae* and *Salmonella typhimurium* (Wiater & Kłopotowski, to be published).

Having expected that affinities of 3-AT and other triazole derivatives for the two 3-AT-sensitive processes may be different and that one of the derivatives might inhibit only the unidentified reaction required for purine synthesis, we undertook the present experiments.

Unexpectedly, it appeared that the inhibitory effects of triazole and TT on growth of *S. typhimurium* can be reversed only by supplementing culture media with serine, cysteine, homocysteine or methionine. Glycine showed the opposite effect by increasing the bacterial growth inhibition. A tentative interpretation of the observed facts assuming an interference of triazole with the metabolism of glycine-derived one-carbon units is proposed. A preliminary report was presented previously (Bogusławski & Kłopotowski, 1966).

[1] Abbreviations used: triazole, 1,2,4-triazole; 3-AT, 3-amino-1,2,4-triazole; AET, 3-β-aminoethyl-1,2,4-triazole; TT, 5-thiol-1,2,4-triazole; DAT, 3,5-diamino-1,2,4-triazole; DMT, 3,5-dimethyl-1,2,4-triazole; DHT, 3,5-dihydroxy-1,2,4-triazole.

MATERIALS AND METHODS

Organisms. The wild strain LT-2 of *S. typhimurium* was obtained from the collection of Dr. B.N. Ames. The auxotrophic mutants WW-3 and WW-14 were obtained in this laboratory by UV-treatment of LT-2 cells, subsequent penicillin selection (Gorini & Kaufman, 1960) and replica plating (Lederberg & Lederberg, 1952). The WW-3 mutant is unable to grow without added glycine and WW-14 requires exogenous serine or glycine for growth.

Media. The minimal medium E of Vogel & Bonner (1956) containing mineral salts, citrate and 0.5% glucose and supplemented (Ames & Garry, 1960) with microelements was used.

Cultures. The bacteria were cultivated with forced aeration by horizontal shaking at 37°. The bacterial growth was followed by measuring light transmittance at 420 mμ of the cultures made in photometer tubes. Each tube contained a glass bead to improve mixing of tube contents. An appropriate calibration curve allowed to convert the photometer readings to micrograms of dry weight per ml. The media were usually inoculated to 25 μg. dry wt. per ml. using bacteria grown in minimal medium supplemented with 1 mM-glycine or 2 mM-DL-serine when necessary, and washed with isotonic saline by centrifugation.

Chemicals. 3-AT was purchased from Fluka AG (Buchs, Switzerland). The other triazole derivatives: TT, AET, DAT, DMT and DHT were kindly supplied by Dr. R.G. Jones from Lilly Research Laboratories, Indianapolis, U.S.A. All

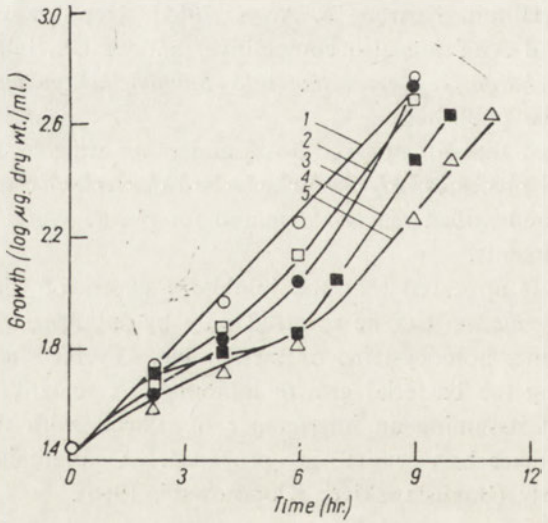


Fig. 1. Effect of triazole derivatives at 2 mM concentration on the growth of the wild strain LT-2 of *S. typhimurium*. Curve *1*, control; curve *2*, 3-AT; curve *3*, DHT; curve *4*, TT; curve *5*, triazole.

other chemicals used were commercial products: L-histidine hydrochloride dihydrate, DL-serine and DL-threonine (Chemapol, Prague, Czechoslovakia), L-cysteine (UCB, Brussels, Belgium), L-arginine, L-leucine, L-isoleucine, L-methionine, L-tryptophan and DL-valine (Reanal, Budapest, Hungary), L-serine and DL-homocysteine (Sigma, St. Louis, U.S.A.), L-tyrosine (British Drug Houses, Poole, Great Britain) and glycine (Polskie Odczynniki Chemiczne, Gliwice, Poland).

RESULTS

Inhibition of bacterial growth by triazole derivatives

The effects of the triazole derivatives present in the culture medium at 2 mM concentrations on growth of LT-2 strain of *S. typhimurium* are shown in Fig. 1. Triazole (the most effective inhibitor), 3-AT, TT and DHT inhibited the growth but the inhibition was transient. Other derivatives: AET, DAT and DMT had no effect. The inhibition began after a period of residual growth and lasted about four hours. The inhibited cells grew at a rate of about 0.17 division per hour, being by 80% slower than the control rate (0.82 division/hr.). After the inhibition phase the growth proceeded at rates of 0.6 to 0.8 division/hr. However, no resistance to triazole could be demonstrated.

Effects of amino acids on the inhibition by triazole

In further experiments the reversal by adenine and histidine of the inhibition produced by triazole, TT or DHT was attempted. However, adenine appeared to increase appreciably the inhibition (see below). Histidine added at high, 5 mM concentration, decreased the inhibition, but only that caused by TT. The growth inhibition produced by triazole or TT could be totally prevented by supplementing culture medium with 0.1 mM-L-serine, L-cysteine or L-methionine (Fig. 2). Since these three amino acids are related to the biosynthesis of methionine, DL-homocysteine and DL-homoserine were also tested. Only the former amino acid was able to prevent the growth inhibitory effect of triazole. Many other amino acids tested were without any detectable effect, namely isoleucine, leucine, tryptophan and valine. L-Tyrosine had a slight reversing effect.

Only one amino acid, glycine, increased the inhibition of bacterial growth by triazole or TT (Fig. 2). The effect of 0.1 mM-glycine was more pronounced than that of equimolar adenine. Even at high concentrations of glycine there was no inhibition by triazole of the growth of the wild strain when 0.1 mM-serine, cysteine or methionine was present in culture medium. At 0.01 mM concentrations cysteine and methionine were still almost fully effective in reversing the effect of 2 mM-triazole and 0.1 mM-glycine. At 0.01 mM concentration serine was only partially effective in the presence of triazole but not at all in media with triazole and glycine. The lower effectiveness of serine may be due to a higher requirement of serine for

bacterial syntheses as compared with those of cysteine and methionine [7 and 22 times higher, respectively, according to our estimates with the use of *S. typhimurium* auxotrophic mutants or 8 and 12 as calculated for *Escherichia coli*, a closely related bacterium (Roberts, Abelson, Cowie, Bolton & Britten, 1957)]. In the absence of

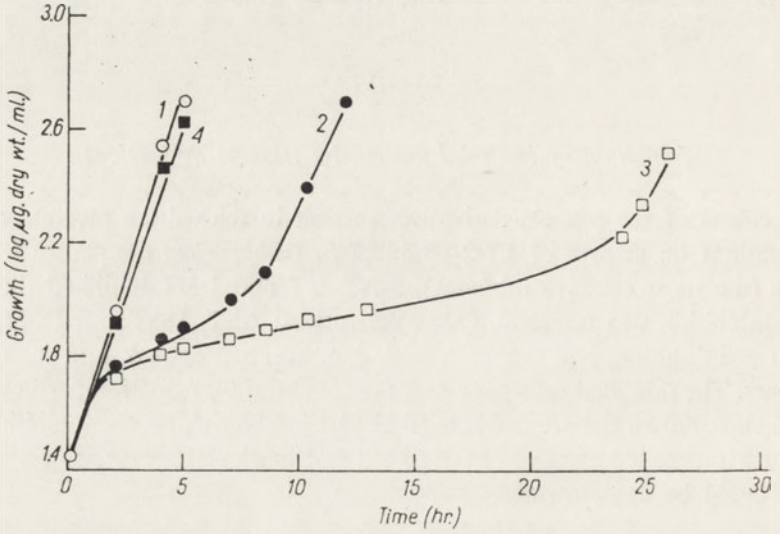


Fig. 2. Effect of amino acids on the inhibition of growth of LT-2 strain of *S. typhimurium* by triazole. Curve *1*, control; curve *2*, 2 mM-triazole; curve *3*, 2 mM-triazole+0.1 mM-glycine; curve *4*, like *2* or *3*, but +0.2 mM-DL-serine, 0.1 mM-cysteine or 0.1 mM-methionine.

triazole glycine did not influence the bacterial growth. The growth inhibitory effect of 20 mM-DHT was not influenced by serine, methionine or glycine. Adenine and, to a lesser extent, cysteine increased the growth lag produced by DHT.

Effects of triazole and amino acids on the growth of glycineless and serineless mutants

The above results suggested that triazole and TT inhibit an enzyme required for biosynthesis of serine and thereby produce a shortage of this amino acid. Since for the biosynthesis of one molecule of cysteine, homocysteine and methionine one molecule of serine is required, these amino acids could act in the inhibited cells by a sparing effect. This supposition was tested in experiments with two auxotrophic mutants, WW-3 and WW-14 which are unable to synthesize glycine and serine, respectively. The metabolic block in WW-3 in most probably in serine hydroxymethyltransferase (EC 2.1.2.1), as in all described mutants requiring for growth exogenous glycine but not serine (Pizer, 1963). The serineless mutant, WW-14 is unable to synthesize serine from 3-phosphoglycerate, because its nutritional requirement can be satisfied by serine or glycine (see below).

One of the experiments with these mutants is depicted in Fig. 3. In the absence of triazole the growth of glycineless mutant WW-3 proceeded only when glycine

had been added to the medium (Fig. 3A, curve *1*). Serine added with glycine (curve *4*) slightly depressed the growth rate in the early phase of culture. In the presence of triazole (Fig. 3B), there was no growth when glycine alone was added to the medium. The addition of serine or methionine restored the growth to the respective control patterns.

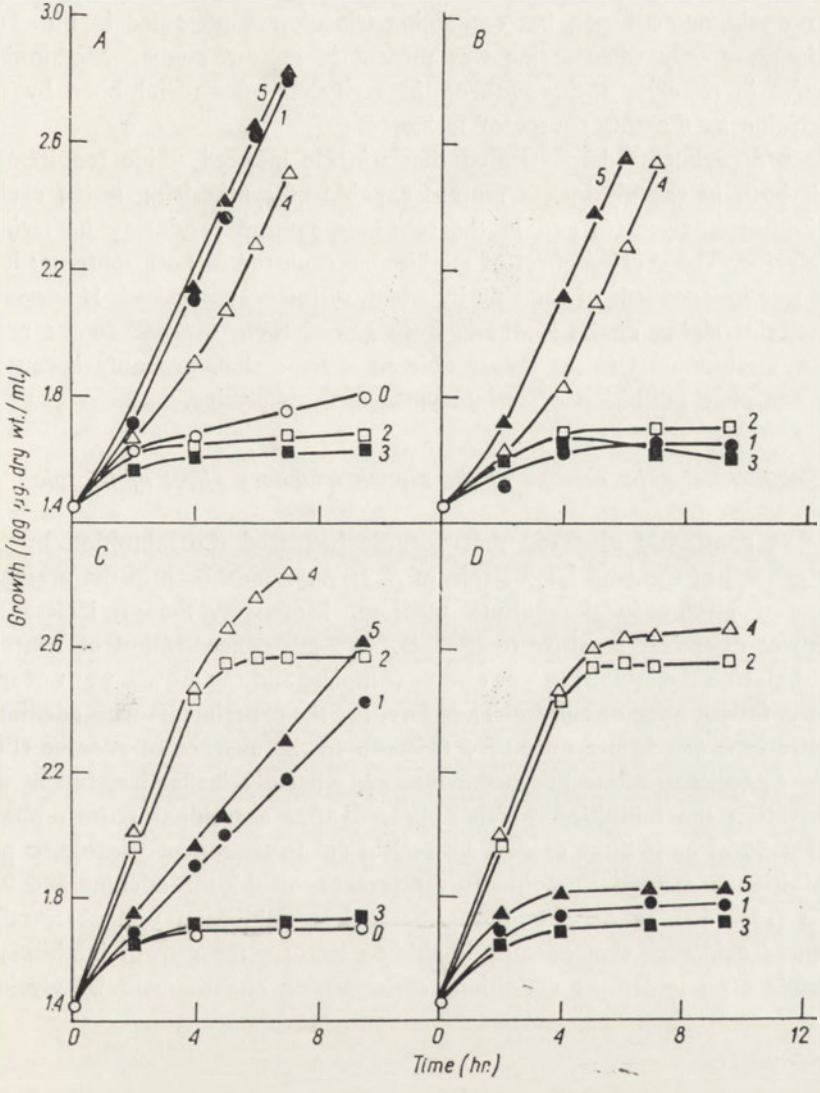


Fig. 3. Effect of serine, methionine and triazole on the growth of *S. typhimurium* mutants. *A* and *B*, the glycineless mutant WW-3; *C* and *D*, the serineless mutant WW-14. *A* and *C*, without triazole; *B* and *D*, with 2 mM-triazole. Curve *0*, no additions; curve *1*, 2 mM-glicyne; curve *2*, 4 mM-DL-serine; curve *3*, 0.2 mM-methionine; curve *4*, 2 mM-glycine+4 mM-DL-serine; curve *5*, 2 mM-glycine +0.2 mM-methionine.

Figure 3C shows that the serineless strain WW-14 grew at the rate of 0.82 division per hour, equal to that of the wild strain LT-2, but only when serine had been added to the medium (curve *2*). The growth proceeded at this rate up to the exhaustion of serine. In the presence of glycine alone (curve *1*) the growth rate was slower by about 60% (0.35 division/hour). The growth was then quite probably limited by the process of serine formation from glycine. The addition of both glycine and methionine resulted in the acceleration of growth by 20% as compared with glycine alone. This indicates that methionine was able to spare serine in this mutant.

The analogous cultures, but containing triazole are presented in Fig. 3D. The bacteria grew only when serine was present in culture media. Methionine was ineffective in restoring the growth of the serineless mutant inhibited by triazole with glycine as the sole source of serine.

These experiments demonstrated that triazole induced serine requirement for growth both in the glycineless mutant capable of synthesizing serine exclusively from 3-phosphoglycerate and in the serineless mutant producing its serine only from glycine. The sparing effect of methionine occurred in both mutants; it means that it was independent of the way by which serine is synthesized. However, when the bacteria relying on its synthesis from glycine were exposed to the action of triazole, methionine was no longer able to spare serine, probably because there was a complete inhibition of serine synthesis from glycine.

Increasing by adenine of the growth inhibitory effect of triazole

It was constantly observed that adenine increased the inhibition by triazole of the growth of the wild LT-2 strain of *S. typhimurium*, both in the presence and absence of glycine in the culture medium. Moreover, the glycineless mutant WW-3 was extremely sensitive to triazole. At 2 mM concentration of triazole and 1 mM of glycine the inhibition was always complete and the growth was not resumed up to the fiftieth hour of culture. Therefore, for the experiments with adenine lower concentrations of triazole were used (Fig. 4). In the absence of adenine (Fig. 4A) triazole at concentrations up to 0.2 mM was virtually ineffective, but at 0.4 mM concentration the inhibition which appeared after a residual growth phase was by about 95%, as if there existed a treshold concentration of triazole to produce an inhibition of bacterial growth. In the presence of 0.2 mM-adenine and 0.1 mM-triazole (Fig. 4B), a definite though transient inhibition was observed. This experiment evidences that adenine is able to increase the growth inhibitory effect of triazole even under the conditions of its strong enhancement by glycine.

DISCUSSION

The experimental data presented in this paper apparently indicate that the unsubstituted triazole and TT inhibit two independent processes of serine biosynthesis. The first one begins with catalysed by NAD-dependent dehydrogenase oxidation of 3-phosphoglycerate yielding 3-phosphohydroxypyruvate. This inter-

mediate undergoes transamination resulting in the second intermediate of serine biosynthesis, 3-phosphoserine. This reaction is probably due to non-specific transaminases, since no mutants unable to conduct it were found (Pizer, 1965). The specific phosphatase produces serine from 3-phosphoserine. The occurrence of this pathway has first been shown by Ichihara & Greenberg (1957) in rat liver, and later in *S. typhimurium* (Umbarger & Umbarger, 1962; Umbarger, Umbarger & Siu, 1963), *E. coli* (Pizer, 1963; Pizer & Potochny, 1964) and mouse brain (Bridgers, 1965). The display of the evidence indicates that in bacteria under physiological conditions serine gives rise to glycine (Roberts, Abelson, Cowie, Bolton & Britten, 1957; Umbarger, Umbarger & Siu, 1963; Pizer, 1965). The reaction is catalysed

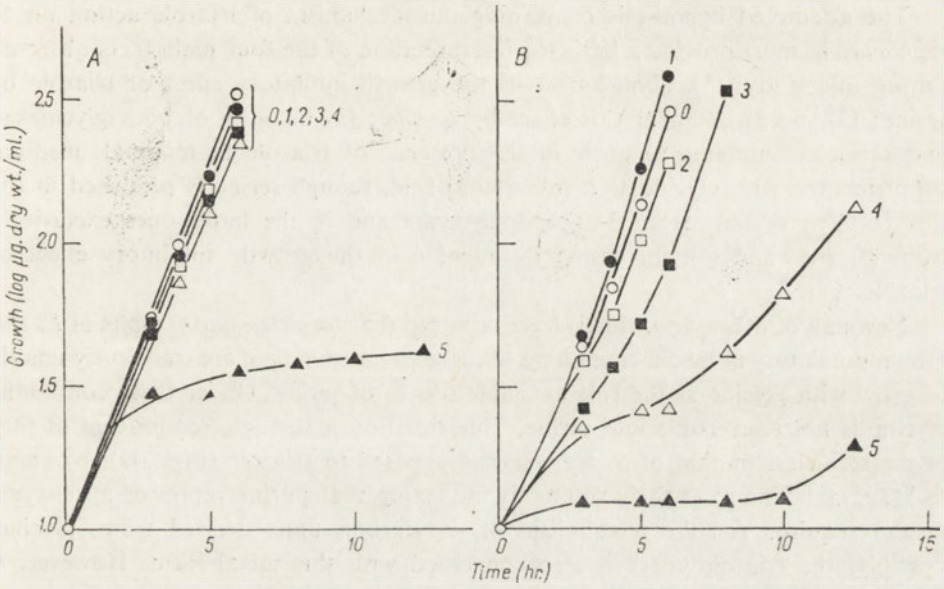


Fig. 4. Increasing effect of adenine on the growth inhibition of the glyciness mutant WW-3 by triazole, in the presence of 2 mM-glycine. *A*, Control, without adenine; *B*, with 0.2 mM-adenine. Curve *0*, without triazole; curve *1*, 0.02 mM-triazole; curve *2*, 0.05 mM-triazole; curve *3*, 0.10 mM-triazole; curve *4*, 0.20 mM-triazole; curve *5*, 0.40 mM-triazole.

by serine hydroxymethyltransferase requiring tetrahydrofolate for accepting one-carbon units. This reaction is of similarly universal occurrence. Its operation in the reverse direction represents the second way of serine biosynthesis. This way is of primary importance at least in serineless mutants unable to form serine by the 3-phosphoglycerate pathway. Possibly, it can furnish serine in the presence of an excess of exogenous glycine. However, in this case one-carbon units, normally supplied by serine, are provided by glycine itself, by a mechanism which remains unknown (Pitts, Stewart & Crosbie, 1961; Newman & Magasanik, 1963; Pizer, 1965).

The two processes of serine biosynthesis are so distinct and devoid of any common

points that an assumption that triazole would inhibit both of them could hardly be envisaged. Nevertheless, *in vitro* experiments have been conducted (K. Krajewska & T. Kłopotowski, unpublished results). They did not demonstrate any inhibitory effect of triazole on 3-phosphohydroxypyruvate reduction or transamination, neither on phosphate cleavage from 3-phosphoserine. The transfer of one-carbon units from serine to 5-amino-4-imidazolecarboxamide-β-ribose-5-phosphate, involving the activity of serine hydroxymethyltransferase, was also proved to be insensitive to triazole. Hence, the inhibition of any enzyme of the either way of serine biosynthesis is not responsible for the inhibition of bacterial growth by triazole. This conclusion is also supported by the fact that none of the three genetic blocks yet found in this metabolic region does result in behaviour resembling the triazole-inhibited bacteria.

The attempted hypothesis concerning the mechanism of triazole action on *S. typhimurium* must provide a basis for interpretation of the four main facts observed during this study: (1), abolishment of the growth inhibitory effect of triazole by serine; (2), potentiation of this effect by glycine; (3) inability of both glycineless and serineless mutants to grow in the presence of triazole in minimal medium supplemented with glycine as a sole amino acid, though serine is produced in the former bacteria only from 3-phosphoglycerate and in the latter ones exclusively from glycine; and (4), increasing by adenine of the growth inhibitory effect of triazole.

Newman & Magasanik (1963) have reported that serineless auxotrophs of *E. coli* grown previously in media containing serine and a purine base are transiently unable to grow with glycine as the sole available source of serine. Under these conditions glycine is not convertible into serine. This situation is strongly reminiscent of that of the serineless mutant of *S. typhimurium* exposed to triazole (Fig. 3D). Newman & Magasanik interpret their results as indicating that purine represses an enzyme system required for the metabolism of one-carbon units derived from glycine. Possibly, the triazole effect is also concerned with this metabolism. However, it does not suffice to explain all the observed facts, namely the apparent requirement for serine by the wild strain bacteria and the enhancement of the triazole effect by glycine. Newman & Magasanik's calculations of one-carbon units requirement by *E. coli* led them to the conclusion that the production of one-carbon units from serine, being their main source, does not satisfy the cell's requirement for these building stones needed for synthesis of purine bases, thymine and some amino acids. The rest must be supplied from the C_2 atom of glycine. It is possible to assume that triazole affects just this process. In this case bacteria need more serine than they can produce [no evidence of derepression of serine biosynthetic enzymes could be found (Umbarger, Umbarger & Siu, 1963)]. The exogenous glycine can aggravate the situation by its feedback inhibition of 3-phosphoglycerate dehydrogenase (Pizer, 1963).

In addition, the enhancement of the triazole effect on bacterial growth by adenine should also be explained by the hypothesis. At first glance, adenine which is known to inhibit its own biosynthesis should spare the one-carbon units and consequently

to reverse the effect of triazole, but the opposite effect was really found. In fact, adenine represses the system generating one-carbon units. The repression is so strong that it outweighs the sparing effect and results in the inhibition of bacterial growth (Dalal, Gots & Gots, 1966). This outweighing could also explain the potentiation of the triazole action by adenine.

Identification of the triazole-sensitive enzyme requires previous elucidation of mechanisms involved in the metabolism of one-carbon units derived from glycine.

This work was supported in part by grant FG-Po-191 from the U.S. Department of Agriculture.

REFERENCES

Ames B.N. & Garry B. (1960). *J. Gen. Microbiol.* **22**, 369.
Bogusławski J. & Kłopotowski T. (1966). *Abstracts 3rd Meeting Fed. Europ. Biochem. Soc., Warsaw*, P. 268.
Bridgers W.P. (1965). *J. Biol. Chem.* **240**, 4591.
Dalal F.R., Gots R.E. & Gots J.S. (1966). *J. Bact.* **91**, 507.
Gorini L. & Kaufman H. (1960). *Science* **131**, 604.
Hilton J.L., Kearney P.C. & Ames B.N. (1965). *Arch. Biochem. Biophys.* **112**, 544.
Ichihara A. & Greenberg D.M. (1957). *J. Biol. Chem.* **224**, 331.
Kłopotowski T. & Wiater A. (1965). *Arch. Biochem. Biophys.* **112**, 562.
Kłopotowski T. & Bagdasarian G. (1966). *Acta Biochim. Polon.* **13**, 153.
Lederberg J. & Lederberg E.M. (1952). *J. Bact.* **63**, 399.
Newman E.B. & Magasanik B. (1963). *Biochim. Biophys. Acta* **78**, 437.
Pitts J.D., Stewart J.A. & Crosbie G.W. (1961). *Biochim. Biophys. Acta* **50**, 361.
Pizer L.I. (1963). *J. Biol. Chem.* **238**, 3934.
Pizer L.I. (1965). *J. Bact.* **89**, 1145.
Pizer L.I. & Potochny M.L. (1964). *J. Bact.* **88**, 611.
Roberts R.B., Abelson P.H., Cowie D.B., Bolton E.T. & Britten T.J. (1957). *Carnegie Inst. Wash. Publ.* **607**, Washington.
Umbarger H.E. & Umbarger M.A. (1962). *Biochim. Biophys. Acta* **62**, 193.
Umbarger H.E., Umbarger M.A. & Siu P.M.L. (1963). *J. Bact.* **85**, 1431.
Vogel H.J. & Bonner D.M. (1956). *J. Biol. Chem.* **218**, 97.

ODWRACANIE PRZEZ SERYNĘ I POTĘGOWANIE PRZEZ GLICYNĘ HAMUJĄCEGO WPŁYWU 1,2,4-TRIAZOLU NA WZROST *SALMONELLA TYPHIMURIUM*

Streszczenie

1. 1,2,4-Triazol hamuje przejściowo wzrost *Salmonella typhimurium*. Inhibicję tę znosi seryna, cysteina, homocysteina lub metionina. Adenina oraz glicyna wzmagają hamujące działanie triazolu.

2. Przypuszczalnie triazol hamuje wytwarzanie jednostek jednowęglowych z glicyny. Wzmaganie efektu triazolu przez glicynę może być wynikiem inhibicji wstecznej biosyntezy seryny. Podobny efekt adeniny może wynikać z reprymowania przez nią enzymów metabolizmu jednostek jednowęglowych.

Received 27 June, 1966

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

S. RAPOPORT and M. MÜLLER

CO_2 FORMATION FROM FORMATE IN RED BLOOD CELLS

Institut für Physiologische und Biologische Chemie der Medizinischen Fakultät der Humboldt-Universität zu Berlin

1. The mechanism of CO_2 formation from formate by rabbit red blood cells was studied using aminotriazole as specific inhibitor of catalase. The inhibition occurs only in the presence of low H_2O_2 and formate concentrations. 2. Both erythrocytes and reticulocytes produce CO_2 from formate under endogenous conditions, which indicates the existence of stationary concentrations of both formate and H_2O_2. The more immature the cell, the lower is the H_2O_2 concentration while that of formate is higher. 3. Inhibition studies with aminotriazole, cyanide and azide indicate that the CO_2 formation proceeds *via* catalase. Data on duck erythrocytes which contain about 0.1% of the catalase activity of rabbit red blood cells, confirm this conclusion.

In reticulocytes there is a considerable formation of CO_2 from [3-^{14}C]serine under endogenous conditions. Observations of Rapoport, Müller & Schultze (1965) indicated that the CO_2 originating from the hydroxymethyl group of serine is formed *via* formate or an intermediate, and in agreement with this a high rate of CO_2 formation from formate was found in reticulocytes.

According to the literature (Nakada & Weinhouse, 1953; Oro & Rappoport, 1959; Aebi, Stocker & Eberhardt, 1963; Venkataraman & Sreenivasan, 1964 and 1966) an oxidation of formate by hydrogen peroxide, mediated by catalase, can be assumed in all cells which contain large amounts of this enzyme, which is well known to possess high activity as formate peroxidase (Nicholls & Schonbaum, 1963). In red blood cells, which are among the richest in catalase, this pathway could be expected to predominate. However, it is an open question whether catalase alone is responsible for the oxidation of formate. Therefore to aid in elucidating the mechanism of CO_2 formation from formate in red blood cells, experiments in which catalase was specifically inhibited were performed.

MATERIAL AND METHODS

The experiments were performed on washed red blood cell suspensions in 0.9% NaCl (haematocrit 50%) from normal rabbits and from those with anaemia produced by repeated withdrawal of blood. The latter suspensions were rich in reticulocytes. The procedures employed were as described previously (Goetze & Rapoport, 1954;

Rapoport *et al.*, 1965). Some experiments were carried out with haemolysates. These were prepared by applying three cycles of freezing and thawing in the presence of nicotinamide at a final concentration of 2 mM; the stroma was removed by 30 min. centrifugation at 3000 *g*. For the experiments on duck erythrocytes washed cell suspensions were prepared in the same manner as described for rabbit red cells.

Conditions of incubation and CO_2 *determination.* The incubations were carried out in Warburg manometers for 60 min. at 38° with air as gas phase. The final volume was 3.0 ml. The inner well contained 0.2 ml. 5% KOH; in the experiments with cyanide the KOH was saturated with it. The incubation was terminated by addition of 1 ml. of 15% trichloroacetic acid. To complete the absorption of the liberated CO_2 the vessels were shaken for a further 30 min. The following substances were employed, at final millimolar concentrations indicated: formate, 2; methylene blue (MB), 0.2; 3-amino-1,2,4-triazole (AT), 80; KCN, 2; azide, 1. Isotopically labelled formate (sp. act. 0.32 mc/m-mole) was used in indicator amounts.

The CO_2 which was collected in KOH was precipitated as $BaCO_3$. The precipitation was carried out at 100° in the following manner. To the KOH solution and washings, about 1.5 ml., were added 1.5 ml. 0.05 M-Na_2CO_3, 0.2 ml. 1.25 M-NH_4Cl, 0.5 ml. 1 M-$BaCl_2$ and 0.5 ml. of absolute ethanol. The gravimetric determination of the $BaCO_3$ and the details of the counting procedure were described previously (Brandt & Rapoport, 1959; Rapoport *et al.*, 1965). Before counting the samples were dried to constant weight in a desiccator over $CaCl_2$. The results were extrapolated to an infinitely thin layer (Gussew, 1957).

The inhibition of catalase by 3-amino-1,2,4-triazole. AT inhibits catalase only in the presence of a small amount of hydrogen peroxide, presumably by reacting with a catalase-H_2O_2 complex (Feinstein, Berliner & Green, 1958; Margoliash & Novogrodsky, 1958; Margoliash, Novogrodsky & Schejter, 1960; Nicholls, 1962). Both lack and excess of the peroxide interfere with the inhibition. Therefore two types of arrangement were employed to furnish optimum amounts of hydrogen peroxide. (1), Diffusion from the side arm of the Warburg flask which was paraffined and contained 0.5 ml. 30% H_2O_2 (Cohen & Hochstein, 1961, 1963). (2), By addition of methylene blue (Warburg, Kubowitz & Christian, 1930). Under such conditions and in the absence of formate, complete inhibition of the catalase both in intact cells and haemolysates was achieved after 60 min. incubation. Determinations of catalase inactivation were checked in the usual manner (Bonnichsen, Chance & Theorell, 1947).

The very low activity of catalase in duck erythrocytes permits only little dilution of the haemolysates for the determination of the enzyme. The permanganometry becomes unfeasible owing to the high unspecific reduction of the permanganate but an exact determination could be performed iodometrically.

RESULTS

CO_2 *formation from formate in intact rabbit red blood cells.* Both erythrocytes and reticulocytes produced CO_2 from formate, the amount being highly variable under endogenous conditions (Fig. 1). The highest CO_2 values for reticulocytes

were somewhat lower than those for erythrocytes (Fig. 2). Addition of methylene blue to erythrocytes yielded CO_2 values which were about three times as high as the low endogenous values but equal to the high ones. It would appear therefore that the variability under endogenous conditions may be due to differences in the endogenous supply of hydrogen peroxide. Addition of an excess of formate increased greatly the formation of CO_2. When both formate and methylene blue were added,

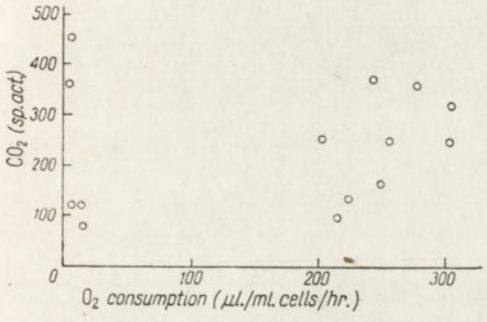


Fig. 1

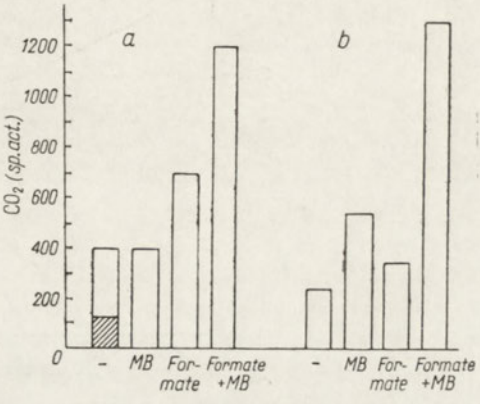


Fig. 2

Fig. 1. CO_2 formation from [^{14}C]formate by rabbit red blood cells under endogenous conditions. The cell suspension was incubated for 60 min. with 2 mM-formate.

Fig. 2. The effect of formate and methylene blue on CO_2 formation from [^{14}C]formate by: (*a*), erythrocytes (the hatched area represents lowest values) and (*b*), reticulocytes. The cell suspension was incubated for 60 min. with 2 mM-formate. Additions: methylene blue, final concn. 0.2 mM; formate, 50 μmoles/3 ml.

CO_2 formation reached values which were tenfold higher than the low endogenous ones. It would appear that in erythrocytes both peroxide and formate are far below saturation, the supply of formate being relatively lower. In reticulocytes methylene blue always caused a greater absolute and relative increase in CO_2 formation, whereas formate had only a small effect. When both formate and methylene blue were present, the same high values were obtained as in erythrocytes. This seems to indicate that in reticulocytes there is a greater supply of formate and a lower level of hydrogen peroxide than in erythrocytes.

It appeared of interest to study the dependence of CO_2 formation on the degree of maturation of the red blood cells. In Fig. 3 is depicted the rate of CO_2 production in the presence of excess formate relative to the oxygen consumption of red blood cells, which may be considered a valid criterion of the degree of maturation. A linear decrease was found which would indicate that the amount of peroxide available is inversely proportional to the intensity of respiration and immaturity of the red

blood cell. In Fig. 4 are represented data on CO_2 formation under endogenous conditions in the presence of methylene blue. Here an increase proportional to the rate of oxygen consumption was found. One may conclude that the amount of formate available increases with the degree of immaturity.

CO_2 formation from formate in haemolysate. In Fig. 5 are shown data on CO_2 formation in haemolysates from erythrocytes and reticulocytes under endogenous

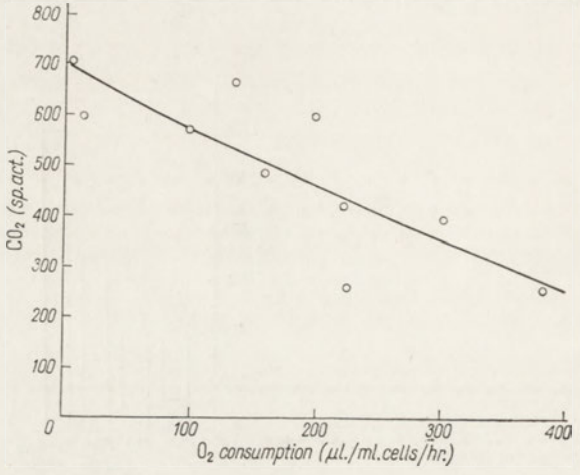


Fig. 3. CO_2 formation from excess [^{14}C]formate in relation to O_2 consumption. To 3 ml. of cell suspension 50 µmoles of formate was added.

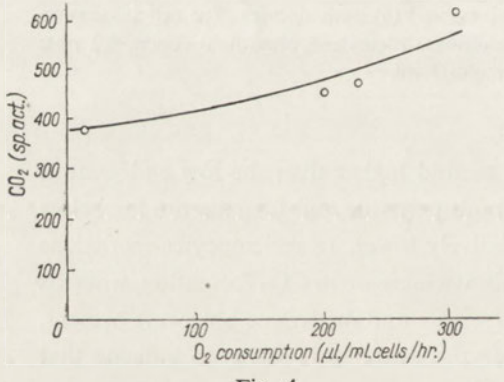


Fig. 4

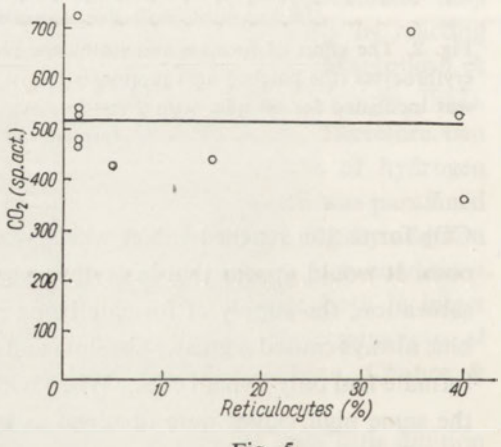


Fig. 5

Fig. 4. CO_2 formation from [^{14}C]formate in red blood cells in the presence of 0.2 mM-methylene blue in relation to O_2 consumption.

Fig. 5. CO_2 formation from [C^{14}]formate in haemolysates of red blood cells in relation to the amount of reticulocytes.

conditions. The values obtained were higher than those for intact cells. Methylene blue had only a minor effect, causing an increase of 8 - 12%. It seems likely, therefore, that in haemolysates the supply of hydrogen peroxide is higher than in intact cells.

The effect of cyanide and azide. It was hoped to elucidate the pathways of CO_2 formation from formate by applying inhibitors of catalase. Table 1 contains data on the influence of cyanide on intact reticulocytes and it may be seen that both respiration and CO_2 formation from formate were strongly inhibited.

Table 1

The effect of cyanide on CO_2 formation from [^{14}C]formate by rabbit reticulocytes

Suspension of reticulocytes was incubated for 2 hr. with 2 mM-formate.

Addition	CO_2		Respiration	
	counts*/mg.	%	µl. O_2/ml. cells	%
None	342	100	326	100
KCN, 2 mM	30	9	65	20

* Corrected to an infinitely thin layer.

Table 2

CO_2 formation from [^{14}C]formate in haemolysates of rabbit red blood cells

Haemolysates were incubated with 2 mM-formate for 1 hr.

Addition	Counts*/mg.			%
	Erythrocytes	Reticulocytes	Average	
None	255; 286	256	266	100
CN^-, 2 mM	46; 44.3	42.3	44	16.5
N_3^-, 1 mM	14.5; 24.8	16.1	18.5	7

* Corrected to an infinitely thin layer.

Table 3

CO_2 formation from [^{14}C]formate by duck erythrocytes

Values are corrected for spontaneous decomposition of the [^{14}C]formate.

Addition	CO_2	
	sp. act.	%
None	8.6	100
Methylene blue, 0.2 mM	3.9	45
Aminotriazole, 80 mM +methylene blue, 0.2 mM	0.7	8

Data from three experiments with haemolysates of erythrocytes and reticulocytes are shown in Table 2. Since the values for erythrocytes and reticulocytes were the same they could be averaged. It may be seen that both cyanide and azide inhibited CO_2 formation to a large extent. The incompleteness of the inhibition was presumably due to two causes: time-dependence of the inhibition, and spontaneous decomposition of the radioactive formate.

In Table 3 are shown the results of an experiment with duck erythrocytes which according to our data contain about 0.1% of the catalase of rabbit red blood cells. The endogenous control values were less than 10% of the low, and less than 2% of the high values of rabbit erythrocytes. Methylene blue caused an inhibition perhaps owing to the inactivating effect of hydrogen peroxide which is observed during catalase determinations. Methylene blue and AT together produced a nearly complete inhibition.

DISCUSSION

Red blood cells are capable of utilizing formate for syntheses as well as of oxidizing it to CO_2. Our results are in agreement with data indicating that red blood cells contain a stationary level of formate, the sources of which are not yet fully known (Annison, 1954). It seems likely that one contribution originates from the hydroxymethyl group of serine which may be metabolized mainly *via* formate (Bertino, Simmons & Donohue, 1962). The mechanism of CO_2 formation from

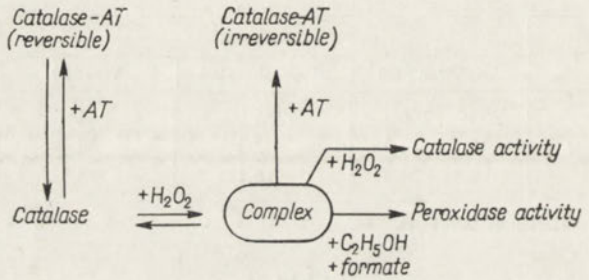


Fig. 6. Scheme of reactions of catalase with 3-amino-1,2,4-triazole.

formate in the red blood cells has not been studied heretofore under physiological conditions. It had appeared likely both from investigations on pure catalase (Nicholls & Schonbaum, 1963) and on catalase-rich tissue extracts (Nakada & Weinhouse, 1953; Oro & Rappoport, 1959; Aebi *et al.*, 1963; Venkataraman & Sreenivasan, 1964, 1966) that formate may be oxidized by catalase in a peroxidase reaction. The specific inhibition by AT would then be expected to influence greatly the formation of CO_2 both from [3-^{14}C]serine and formate. The supposed mechanism of the action of AT is depicted in Fig. 6. AT inhibits catalase only in the presence of optimal low hydrogen peroxide concentrations (Feinstein *et al.*, 1958; Margoliash & Novogrodsky, 1958; Margoliash *et al.* 1960; Nicholls, 1962). Catalase forms with peroxide a complex which is decomposed either by hydrogen donors such as ethanol and formate, or by excess of the peroxide (Nicholls & Schonbaum, 1963). AT competes with these compounds in an irreversible reaction. The level of hydrogen peroxide in red blood cells is presumably so low that the steady-state concentration of the

catalase-H_2O_2 complex is below that needed for combination with AT, and complete inhibition of catalase is achieved by providing additional peroxide. By adding excessive amounts of hydrogen peroxide or with larger amounts of formate, the steady-state concentration of complex I is lowered to such an extent that inhibition by AT does not occur.

As indicated in the previous section, the concentration of hydrogen peroxide is lower in reticulocytes than in erythrocytes. Since there is no large difference in the activity of flavoproteins in the hyaloplasma (Rapoport & Wagenknecht, 1957) which might yield hydrogen peroxide, it would seem reasonable to assume that the reticulocyte by virtue of its greater oxidative metabolism utilizes the peroxide by as yet unknown pathways. No definite statement can be made with regard to the concentration of formate. It appears likely that the rate of formation is much greater in the reticulocyte while the demand for C_1-units for synthesis of purines and other compounds is also higher.

The formation of CO_2 from formate is independent of the respiratory chain as indicated by its occurrence in erythrocytes and haemolysates, which both lack the respiratory system. The large decline of CO_2 production after specific inhibition of catalase by AT both in intact cell and haemolysates leads to the conclusion that catalase is mainly responsible for CO_2 formation. The results with cyanide and azide are confirmatory.

Red blood cells of persons with acatalasia, which contain only 0.1 - 1.3% of catalase compared with normal blood, are supposed to show no disturbance in CO_2 formation from formate (Aebi, Baggiolini, Dewald, Lauber, Suter, Micheli & Frei, 1964). One must assume that either even minimal amounts of catalase suffice for formate oxidation or that there exists another as yet undetected possibility for formate oxidation. Duck erythrocytes which contain about 0.1% of the catalase of rabbit red blood cells show a much lower but still definite rate of CO_2 formation from formate which is one or two orders of magnitude greater than the relative catalase activity. The strong inhibition by the combined action of methylene blue and AT would indicate, however, that even the low CO_2 production of these cells proceeds entirely *via* catalase.

REFERENCES

Aebi H., Baggiolini M., Dewald B., Lauber E., Suter H., Micheli A. & Frei J. (1964). *Enzymol. biol. clin.* **4**, 121.

Aebi H., Stocker F. & Eberhardt M. (1963). *Biochem. Z.* **336**, 526.

Annison E.A. (1954). *Biochem. J.* **58**, 670.

Bertino J.R., Simmons B. & Donohue D.M. (1962). *J. Biol. Chem.* **237**, 1314.

Bonnichsen R.K., Chance B. & Theorell H. (1947). *Acta Chem. Scand.* **1**, 685.

Brandt H. & Rapoport S. (1959). *Acta biol. med. germ.* **2**, 290.

Cohen G. & Hochstein P. (1961). *Science* **134**, 1756.

Cohen G. & Hochstein P. (1963). *Biochemistry* **2**, 1420.

Feinstein R.N., Berliner S. & Green F.O. (1958). *Arch. Biochem. Biophys.* **76**, 32.

Goetze E. & Rapoport S. (1954). *Biochem. Z.* **326**, 53.

Gussew N.G. (1957). *Leitfaden für Radioaktivität u. Strahlenschutz.* Verlag Technik. Berlin.
Margoliash E. & Novogrodsky A. (1958). *Biochem. J.* **68**, 468.
Margoliash E., Novogrodsky A. & Schejter A. (1960). *Biochem. J.* **74**, 339.
Nakada H.J. & Weinhouse S. (1953). *Arch. Biochem. Biophys.* **42**, 257.
Nicholls P. (1962). *Biochim. Biophys. Acta* **59**, 414.
Nicholls P. & Schonbaum G.R. (1963). *The Enzymes*, vol. 8, p. 147. Academic Press, New York.
Oro J. & Rappoport D.A. (1959). *J. Biol. Chem.* **234**, 1661.
Rapoport S., Müller M. & Schultze M. (1965). *Acta Biol. med. germ.* **15**, 209.
Rapoport S. & Wagenknecht C. (1957). *Z. physiol. Chem.* **309**, 196.
Venkataraman S. & Sreenivasan A. (1964). *Experientia* **20**, 370.
Venkataraman S. & Sreenivasan A. (1966). *Enzymologia* **28**, 91.
Warburg O., Kubowitz F. & Christian W. (1930). *Biochem. Z.* **227**, 245.

WYTWARZANIE CO_2 Z MRÓWCZANU W CZERWONYCH KRWINKACH

Streszczenie

1. Badano mechanizm wytwarzania CO_2 z mrówczanu przez czerwone krwinki królika przy zastosowaniu aminotriazolu jako swoistego inhibitora katalazy. Inhibicja zachodzi tylko w obecności małych stężeń H_2O_2 oraz mrówczanu.

2. Zarówno erytrocyty jak i retikulocyty wytwarzają CO_2 z mrówczanu w warunkach endogennych, co wskazuje na obecność stałego poziomu zarówno mrówczanu jak i H_2O_2. Im mniej dojrzałe są komórki, tym mniejsze jest stężenie H_2O_2 zaś wyższe stężenie mrówczanu.

3. Badania z użyciem takich inhibitorów jak aminotriazol, cyjanek oraz azydek wskazują, że w wytwarzaniu CO_2 z mrówczanu bierze udział katalaza. Dane uzyskane przy badaniu krwinek kaczki, zawierających zaledwie 0.1% aktywności katalazy krwinek królika, potwierdzają to przypuszczenie.

Received 15 July, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

T. CHOJNACKI, ANNA RADOMIŃSKA-PYREK and T. KORZYBSKI

ON THE FEATURES OF CYTOSINE RING REQUIRED FOR THE COENZYMIC FUNCTION OF CYTIDINE NUCLEOTIDES IN BIOSYNTHESIS OF PHOSPHOLIPIDS

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

This paper is dedicated with respect and affection to Prof. Dr. Józef Heller, the discoverer of insect inorganic pyrophosphates that led to our studies on biosynthesis of phospholipids

1. Four pyrimidine analogues of cytidine diphosphate choline have been chemically prepared and used as precursors for phospholipid biosynthesis in homogenates of rat brain and liver. 2. The rate of the biosynthesis was followed by using ^{32}P-label introduced into β-phosphorus atom of the above nucleoside diphosphates. 3. The following features of cytidine pyrimidine ring were found to be important for the precursor function: unsubstituted NH_2 at C-6, 4,5 double bound, no substitution with CH_3 at C-5. Thus the fragment $=\overset{6}{C}(NH_2)—\overset{5}{C}H=\overset{4}{C}H—$ is of crucial importance.

The lack of precursor activity in phospholipid biosynthesis of analogues of cytidine diphosphate choline containing other natural purines and pyrimidines (adenine, guanine, uracil and inosine) instead of cytosine has been demonstrated by Kennedy & Weiss (1956). In the previous study (Chojnacki & Korzybski, 1963) one artificial analogue of cytidine diphosphate choline containing 6-*N*-methyl cytosine was also found to be deprived of coenzyme function in biosynthesis of phospholipids.

Looking for some elements in the structure of cytosine that might have crucial importance enabling the coenzyme to perform its role some other analogues of cytidine coenzyme were synthetized and their effectiveness was checked.

The results of these experiments are presented in this paper; they include studies with analogues (II, III, IV and V) containing 5-methylcytidine, 4,5-dihydrocytidine, 4,6-(bis)-hydroxylamino-4,5-dihydro-2-ketopyrimidine riboside and 6-hydroxylamino-2-ketopyrimidine riboside, respectively (see Fig. 1).

A preliminary report on the results with the first two analogues (II and III) has already been presented (Chojnacki & Korzybski, 1966).

MATERIALS

Choline chloride was a Light & Co. Ltd. (Colnbrook, England) product, 2-monoethylaminoethanol and dicyclohexylcarbodiimide were BDH (Poole, England) products, cytidine-5-monophosphoric acid was a CalBiochem (Los Angeles, Calif., U.S.A.) product. Hydroxylamine hydrochloride was a commercial preparation which was recrystallized several times from ethanol to a constant melting point, 152 - 153°. ^{32}P-labelled orthophosphoric acid was supplied by the Division of Isotope Distribution (Institute for Nuclear Research, Warsaw). We are indebted to Doc. Dr. W. Szer from our Institute for a sample of 5-methylcytidine-5′-monophosphoric acid.

EXPERIMENTAL

Phosphorylcholine and phosphorylmonomethylaminoethanol labelled with ^{32}P were prepared according to Riley (1944) and purified on ion exchange columns as described by Ansell & Chojnacki (1966). Cytidine diphosphate choline (I) labelled in β-P atom with ^{32}P was prepared according to Kennedy (1956) from [^{32}P]phosphorylcholine and CMP.

[β-^{32}P]Cytidine diphosphate monomethylaminoethanol (Ia) was prepared from [^{32}P]phosphorylmonomethylaminoethanol and CMP using a similar procedure (Kennedy & Weiss, 1956; Ansell & Chojnacki, 1966).

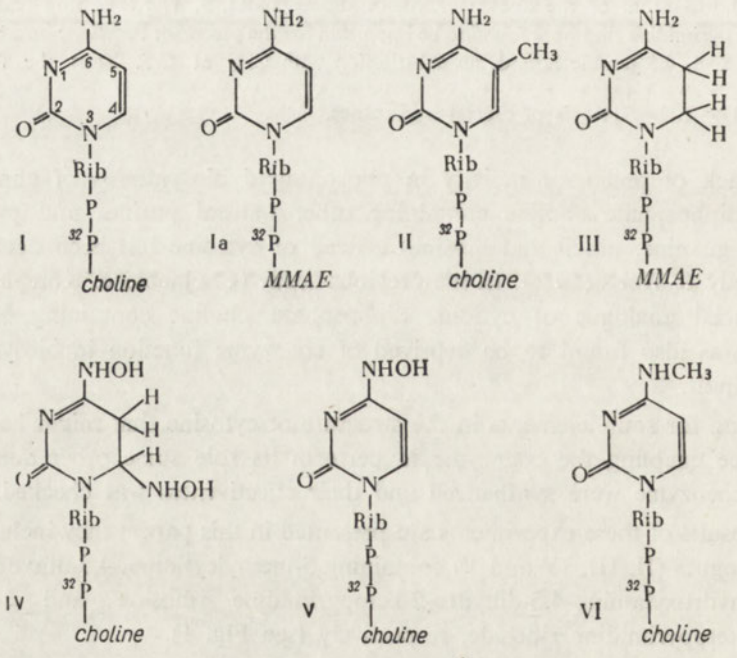


Fig. 1. Analogues of cytidine diphosphate choline. Rib, ribose; P and ^{32}P, phosphate groups; *MMAE*, monomethylaminoethanol.

5-Methylcytidine diphosphate choline (II) labelled in β-P atom with ^{32}P was prepared by the method of Kennedy (1956) from 5-methylcytidine-5′-monophosphoric acid and [^{32}P]phosphorylcholine. The product had the ratio of UV extinction to phosphorus, 1 : 2.08, based on the molecular extinction value of $\varepsilon_{287} = 14.3$ for 5-methylcytidine-5′-monophosphoric acid (theory, 1 : 2) and exhibited on paper chromatography and acid hydrolysis (Chojnacki & Korzybski, 1963) identical patterns as cytidine diphosphate choline.

[β-^{32}P]4,5-Dihydrocytidine diphosphate monoethylaminoethanol (III) was prepared by hydrogenation of Ia with the rhodium-on-alumina catalyst according to Cohn & Doherty (1956) and Green & Cohen (1957a, b) as described by Janion & Shugar (1960) during 2.5 hr. The characteristics of the resulting products are discussed in the following section.

[β-^{32}P]4,6-(bis)-Hydroxylamino-4,5-dihydro-2-ketopyrimidine riboside-5′-diphosphate choline (IV) was prepared from I by reaction with hydroxylamine (2 M final concentration) at pH 6.5 during 6 hr. at 37° according to Janion & Shugar (1965). The characteristics of this and next compounds are discussed in the following section.

[β-^{32}P]6-Hydroxylamino-2-ketopyrimidine riboside-5′-diphosphate choline (V) was prepared from IV by its acidification that led to the acid-catalysed elimination of hydroxylamine with reformation of the 4,5 double bond according to Janion & Shugar (1965).

A Unicam SP 500 spectrophotometer was used for all measurements. The assays of ^{32}P and phosphorus and details of experiments with tissue homogenates were described previously (Chojnacki & Korzybski, 1963; Ansell & Chojnacki, 1966).

RESULTS

The absorption spectra of Ia and III are presented in Fig. 2. Within 2.5 hr. of hydrogenation complete disappearance of cytosine peak at 281 mμ could not be obtained, the remainder indicated the presence of about 20% of unaltered cytidine coenzyme. This could not be lowered as the extending of time of hydrogenation led also to a decrease of the absorption in the 240 mμ region characteristic for dihydroderivatives. The incubation of tissue homogenates with III was performed immediately after hydrogenation, the catalyst having been removed by centrifuging. The details of incubation and the assay of incorporation of ^{32}P into phospholipids were described previously (Chojnacki & Korzybski, 1963).

Upon incubating homogenates of liver or brain of the rat with III the amount of ^{32}P incorporated into phospholipids was about 4 times less than that obtained with unaltered coenzyme (I). This may suggest that III was inactive as coenzyme; low incorporation that was observed should be accounted for by the presence of some 20% of unaltered coenzyme in the hydrogenated mixture (Table 1, expt. 1).

The results with II clearly indicate that the 5-methylcytidine can not replace cytidine in the coenzyme. The incorporation of ^{32}P into phospholipids from II was practically absent (Table 1, expt. 2).

Table 1

The effectiveness of analogues of cytidine coenzymes as precursors in biosynthesis of phospholipids

Each incubation sample (1.5 ml.) contained 100 mg. of homogenized tissue in medium containing: 31.6 mM-KCl; 9.5 mM-KF; 20 mM-$MgCl_2$; 4 mM-Na_2HPO_4; 26.6 mM-tris-HCl, pH 7.4 and 0.05 μmole of the respective labelled nucleotide. Incubation, 1 hr. at 37°. All incubation samples of expt. 3 contained in addition 16 mM-hydroxylamine. Specific activity of labelled nucleotides was from 3×10^5 to 8×10^5 counts/min./μmole. The incorporation of ^{32}P was measured as described previously (Chojnacki & Korzybski, 1963). It is expressed as per cent of added labelled precursor ±S.D. In parentheses the number of determinations.

Expt.	Type of analogue	Symbol	^{32}P incorporated into phospholipids (%)	
			Liver	Brain
1	Cytidine diphosphate monomethyl-aminoethanol	Ia	32.2	25.0; 27.6
	4,5-Dihydrocytidine diphosphate mono-methylaminoethanol	III	7.6±0.4 (4)	7.2; 8.1
2	Cytidine diphosphate choline	I	47.1; 30.5	35.8; 18.7
	5-Methylcytidine diphosphate choline	II	0.12±0.1 (3)	0.27±0.1 (3)
3	Cytidine diphosphate choline	I	53.8±2.5 (4)	29.4
	4,6(bis)-Hydroxylamino-4,5-dihydro-2-ketopyrimidine riboside-5′-diphosphate choline	IV	6.1±0.5 (4)	5.5; 5.4
	6-Hydroxylamino-2-ketopyrimidine riboside-5′-diphosphate choline	V	6.6±0.2 (4)	5.2; 4.3

Figure 3 represents the absorption spectra of the products of the reaction of I with hydroxylamine and with hydrochloric acid. The absorption spectra of unaltered cytidine diphosphate choline at pH 1 and 7.4 were given for comparison.

The reaction of about 2 μmoles of cytidine diphosphate choline (I) in 1 ml. of 2 M-hydroxylamine at pH 6.5 at 37° resulted in the disappearance of the absorption peak of cytosine within 6 hr. This was checked by taking 0.1 ml. samples from the mixture after 2, 4 and 6 hr., diluting them to 5 ml. with 0.05 M-tris-HCl buffer, pH 7.4 and estimating the absorption spectrum (Fig. 3). The samples taken after 6 hr. and diluted to 5 ml. with 0.05 M-tris-HCl buffer, pH 7.4, that contained IV as judged by their absorption spectrum (cf. Janion & Shugar, 1965) were used as such for experiments with tissue homogenates.

Another 0.1 ml. sample of the above mentioned reaction mixture taken after 6 hr. was diluted to 5 ml. with 0.1 N-HCl and left at room temperature for 20 hr. After this time the absorption spectrum was measured. It revealed the appearance of an absorption peak in 280 mμ region (Fig. 3). The height of this maximum indicates that a large percentage of 4,5 double bonds originally present in cytidine diphosphate choline that had disappeared upon incubation with hydroxylamine at pH 6.5 was restored during the subsequent acid-catalysed elimination of hydroxyl-

amine with reformation of 4,5 double bond. The ε values of IV and V being not known, exact calculation of restoration percentage could not be done. The sample in 0.1 N-HCl that contained V as judged by its absorption spectrum (cf. Janion & Shugar, 1965) was adjusted to pH 7 with 1 N-NaOH and used as such for experiments with tissue homogenates.

The treatment of cytidine diphosphate choline (I) with 2 M-hydroxylamine followed by the treatment with 0.1 N-HCl did not affect the pyrophosphate bond of the coenzyme as revealed by charcoal absorption tests, wherein all the label localized in β-P atom was absorbed. The phosphorylcholine moiety of the coenzyme has also not been affected as shown by paper chromatography of V and of its acid hydrolysis products.

In expt. 3 of Table 1 both modified coenzymes (IV and V) in liver and brain homogenates were found to be much less active than I as precursors of phospholipids.

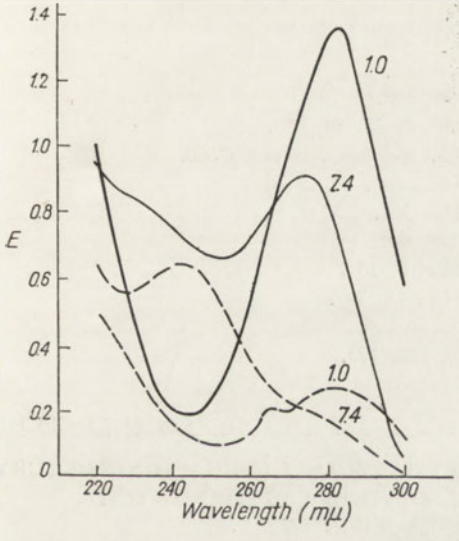


Fig. 2

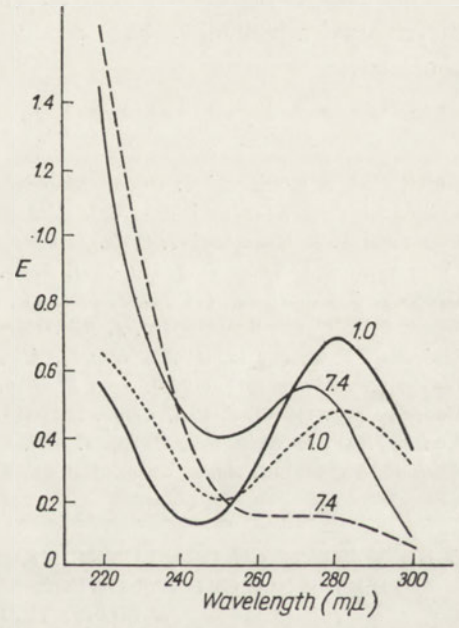


Fig. 3

Fig. 2. Absorption spectra of (——), cytidine diphosphate monomethylaminoethanol (Ia) and (— — —), of its hydrogenated derivative (III) at pH values 1.0 (0.1 N-HCl) and 7.4 (0.05 M-tris-HCl).

Fig. 3. Absorption spectra of (——), cytidine diphosphate choline (I); (— — —), 4,6-(bis)-hydroxylamino-4,5-dihydro-2-ketopyrimidine riboside-5′-diphosphate choline (IV) and (- - - - -) 4-hydroxylamino-2-ketopyrimidine riboside-5′-diphosphate choline (V) at pH values 1.0 (0.1 N-HCl) and 7.4 (0.05 M-tris-HCl).

The incubations of I with both tissue homogenates were in this case performed in the presence of 16 mM-hydroxylamine in order to rule out any effect of hydroxylamine itself on the rate of phospholipids formation. This was the same concentration as that brought in when adding samples of IV and V to the homogenates. Hydroxyl-

amine was found to result in only negligible decrease of the formation of labelled phospholipids from cytidine diphosphate choline when compared with results obtained without the hydroxylamine supplement. The fact that a small incorporation of ^{32}P from IV and V into phospholipids did occur might have been due to the presence of a small amount of unaltered I in the preparations.

The results obtained are in agreement with the previous data (Chojnacki & Korzybski, 1963) on the effect of *N*-substitution at C-6 (VI) and suggest that the $=\overset{6}{C}(NH_2)-\overset{5}{C}H=\overset{4}{C}H-$ fragment of cytosine is of crucial importance for coenzymic activity of cytidine nucleotides.

The help of Doc. Dr. W. Szer and Dr. C. Janion in preparing some of the pyrimidine analogues is greatly appreciated.

Warning for the workers using dicyclohexylcarbodiimide: In one of us the use of dicyclohexylcarbodiimide had produced diffuse eczematic changes of allergic character.

REFERENCES

Ansell G.B. & Chojnacki T. (1966). *Biochem. J.* **98**, 303.
Chojnacki T. & Korzybski T. (1963). *Acta Biochim. Polon.* **10**, 455.
Chojnacki T. & Korzybski T. (1966). III[rd] *F.E.B.S. Meeting*, Abstract F160.
Cohn W.E. & Doherty D.G. (1956). *J. Amer. Chem. Soc.* **78**, 2863.
Green S. & Cohen S.S. (1957a). *J. Biol. Chem.* **225**, 397.
Green S. & Cohen S.S. (1957b). *J. Biol. Chem.* **228**, 601.
Janion C. & Shugar D. (1960). *Acta Biochim. Polon.* **7**, 309.
Janion C. & Shugar D. (1965). *Acta Biochim. Polon.* **12**, 337.
Kennedy E.P. (1956). *J. Biol. Chem.* **222**, 185.
Kennedy E.P. & Weiss S.B. (1956). *J. Biol. Chem.* **222**, 193.
Riley R.F. (1944). *J. Amer. Chem. Soc.* **66**, 512.

O NIEODZOWNYCH DLA FUNKCJI KOENZYMATYCZNEJ CECHACH STRUKTURY PIERŚCIENIA CYTOZYNOWEGO NUKLEOTYDÓW CYTYDYNOWYCH W BIOSYNTEZIE FOSFOLIPIDÓW

Streszczenie

1. Otrzymano na drodze syntezy chemicznej cztery pirymidynowe analogi cytydynodwufosfocholiny. Zastosowano je w homogenatach mózgu i wątroby szczura jako prekursory fosfolipidów.

2. Szybkość biosyntezy fosfolipidów oznaczono używając ^{32}P wprowadzonego do β-atomu fosforu w wyżej wymienionych analogach nukleozydodwufosforanów.

3. Dla funkcji prekursorowej nieodzowne są następujące cechy struktury pierścienia pirymidynowego: niesubstytuowana grupa aminowa przy C-6, podwójne wiązanie przy C-4, C-5, brak substytucji grupą metylową wodoru przy C-5. Fragment $=\overset{6}{C}(NH_2)-\overset{5}{C}H=\overset{4}{C}H-$ cytozyny ma podstawowe znaczenie.

Received 20 July, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

WANDA MEJBAUM-KATZENELLENBOGEN, ELEONORA WIECZOREK
and CH. MASKOS

SIALIC ACID IN RABBIT KIDNEY

Department of Physiological Chemistry, Faculty of Pharmacy, Medical School, and Department of Biochemistry, University, Wrocław

1. For removing the substances interfering with sialic acid determination, two procedures (with tannin and sulphosalicylic acid) were elaborated. 2. Per 100 g. of rabbit kidney, 70 mg. of sialic acid was found, mainly in the nuclear-mitochondrial fraction.

Free sialic acid has not been found in animal tissues but it is known to be present in sialoproteins, giving them the acidic character, and in sialomucopolysaccharides. In blood serum, sialic acid is present in the sulphosalicylic acid-soluble protein fraction. However, the sulphosalicylic acid-soluble proteins from the kidney of rabbit and some other mammals contain only very small amounts of hexose and no sialic acid (Mejbaum-Katzenellenbogen & Wieczorek, 1966), indicating that the kidney sialic acid is bound to the sulphosalicylic acid-insoluble fraction.

Brunngraber & Brown (1963, 1964) demonstrated the presence of sialomucopolysaccharides in mitochondria and microsomes of brain tissue. From kidney and liver mitochondria Rzeczycki, Grudzińska & Hillar (1962) isolated a basic (cathodic) protein present in the sulphosalicylic acid-soluble fraction. It is not known whether sialoproteins or sialomucopolysaccharides are able to form with basic proteins salt-like compounds but, if so, sialic acid should predominate in subcellular particles. In the present work an attempt was made to determine the distribution of sialic acid in subcellular fractions of rabbit kidney.

Reliable estimation of sialic acid is difficult to achieve and requires initial purification of the material to remove substances interfering with the determinations, e.g. sucrose used for fractional centrifugation, deoxyribose present in nucleic acid. Therefore two procedures, using tannin and sulphosalicylic acid, were elaborated for initial isolation and purification of proteins from sucrose homogenate and subcellular fractions.

MATERIALS AND METHODS

Reagents used: 2-thiobarbituric acid (Schuchardt, München, Germany); diphenylamine, purified twice by crystallization from ethanol; cyclohexanone (Fabryka

Odczynników Chemicznych, Gliwice, Poland); sulphosalicylic acid (Unia, Warszawa, Poland); tannin (Politechnika Śląska, Gliwice, Poland, batch no. 3-TO/60, or imported from U.S.S.R.); and pentadigalloyloglucose obtained by purification of commercial tannin preparations by the method of Armitage *et al.* (1961).

Homogenate and subcellular fractions. Rabbits were killed by bleeding, the kidneys excised and kept for 15 min. at -6°. Then the connective and fat tissues as well as the capsula and renal pelvis were removed, and the kidneys were cut into about 0.5 cm. thick slices. These were disintegrated with a tenfold volume of 0.25 M-sucrose containing 1.8 mM-$CaCl_2$ in a Waring Blendor type homogenizer during 2 min. (in several cycles). The homogenate was separated by centrifuging into a nuclear-mitochondrial fraction (20 000 *g* sediment) and a microsomal-cytoplasmic fraction (20 000 *g* supernatant).

For purification of the homogenate and subcellular fractions from sucrose and other compounds interfering in sialic acid determination, two procedures were elaborated.

I. Purification with tannin. To 10 g. of the kidney homogenate was added 0.2 g. of tannin dissolved in a small amount of water. The same amount of tannin was added to preparations of either of the subcellular fractions obtained from 10 g. of the homogenate. The sample was mixed and after 30 min. at room temperature the protein-tannin precipitate was centrifuged at 3500 rev./min. for 15 min. and washed with 0.5% tannin solution until sugar-free (α-naphthol test). The washed sediment was freed of tannin by washing with acetone. Traces of acetone were evaporated by a stream of cold air, then the sediment was suspended in 10 ml. of 0.15 M-sulphosalicylic acid and heated for 30 min. in a boiling water bath in tubes provided with air coolers. After cooling in tap water, the hydrolysate was filtered and sialic acid was determined.

It was found that the same amount of sialic acid was liberated by 30-min. hydrolysis in 0.15 M-sulphosalicylic acid as by 1-hr. hydrolysis in 0.1 N-H_2SO_4 used by Warren (1959).

II. Purification with sulphosalicylic acid. To 10 g. of the homogenate and the microsomal-cytoplasmic fraction was added 10 ml. of 0.3 M-sulphosalicylic acid, and the nuclear-mitochondrial sediment was suspended in 10 ml. of 0.15 M-sulphosalicylic acid. The samples were centrifuged at 3500 rev./min., and the supernatant was collected for further determinations. The sediment of precipitated protein was washed with 0.15 M-sulphosalicylic acid until sugar-free, then washed twice with acetone; traces of acetone were removed by a stream of cold air, the residue suspended in 10 ml. of 0.15 M-sulphosalicylic acid and hydrolysed.

In the supernatants collected after sulphosalicylic acid treatment the amount of acid-soluble protein was determined, then the protein was precipitated with a 20-fold excess of tannin and centrifuged. The protein-tannin sediment was washed with 0.5% tannin solution until sugar-free, then tannin was removed with acetone. Traces of acetone were evaporated. The sediment was dissolved in 0.1 N-NaOH, estimated for protein content and submitted to hydrolysis in 0.15 M-sulphosalicylic acid.

Analytical methods. In the sulphosalicylic acid hydrolysate, sialic acid was determined by the diphenylamine method of Seifer & Gerstenfeld (1959) and the thiobarbituric acid method of Warren (1959), *N*-acetylneuraminic acid (Calbiochem, Lucerne, Switzerland) being used as standard. Protein was determined by the tannin micromethod according to Mejbaum-Katzenellenbogen (1955).

RESULTS

The efficiency of purification of sucrose homogenates and subcellular fractions by the tannin and sulphosalicylic acid procedures was checked by two methods commonly applied for sialic acid estimation: the diphenylamine method of Seifer & Gerstenfeld (1955) and a specific thiobarbituric acid method of Warren (1959). The result of a typical determination is presented in Table 1.

Table 1

Determination of sialic acid in homogenates and subcellular fractions of rabbit kidney after purification by tannin and sulphosalicylic acid

Procedure I: Tannin was added to sucrose homogenate or subcellular fraction. The protein-tannin sediment was washed with tannin solution, then tannin removed with acetone. The sediment was hydrolysed in 0.15 M-sulphosalicylic acid for 30 min. on a boiling water bath.

Procedure II: Sulphosalicylic acid was added to sucrose homogenate or subcellular fraction to 0.15 M concn. The sediment after washing with the acid and acetone was hydrolysed.

In the hydrolysates, sialic acid was determined by the thiobarbituric acid method of Warren (1959) and the diphenylamine method of Seifer & Gerstenfeld (1955).

Material	Sialic acid (mg. per 100 g. fresh tissue)			
	Procedure I		Procedure II	
	Thiobarbituric acid	Diphenylamine	Thiobarbituric acid	Diphenylamine
Whole homogenate	76.0	84.0	47.0	46.0
Nuclear-mitochondrial fraction	41.8	47.1	34.75	39.7
Microsomal-cytoplasmic fraction	30.0	31.5	11.0	14.0

For the kidney homogenate and either fraction studied, the values obtained by the two methods were in good agreement indicating the removal of substances interfering in the diphenylamine method.

After purification with sulphosalicylic acid, the amounts of sialic acid found in the whole homogenate and the microsomal-cytoplasmic fraction were by about 30 - 40% smaller than after purification with tannin. No significant differences were found in the content of sialic acid in the nuclear-mitochondrial fraction after purification by the tannin or sulphosalicylic acid procedures.

Distribution of sialic acid and sulphosalicylic acid-soluble proteins in subcellular fractions of rabbit kidney

As the methods of Warren (1959) and Seifer & Gerstenfeld (1955) gave practically the same results, only the latter one was used for experiments with the isolated subcellular fractions. The material to be tested was initially purified with tannin and acetone or with sulphosalicylic acid and acetone. The average values from 13 determinations are summarized in Table 2. After purification by the tannin procedure, the content of sialic acid amounted to 70 mg. per 100 g. of fresh kidney. The nuclear-mitochondrial fraction contained 70% of the total, and the remaining 30% was present in the protein of the cytoplasm and microsomes. At variance with sialic acid, the sulphosalicylic acid-soluble proteins originated only in 30% from the nuclear-mitochondrial fraction, and in 70% from the microsomal-cytoplasmic fraction. In those proteins no sialic acid was found.

Table 2

Distribution of sialic acid in subcellular fractions of rabbit kidney

Procedure I: purification with tannin; procedure II: purification with sulphosalicylic acid. Sialic acid was determined by the diphenylamine method of Seifer & Gerstenfeld (1955). The results are averages of 13 assays, ±S.D., calculated per fresh tissue weight.

Material	Sialic acid				Sulphosalicylic acid-soluble protein	
	Procedure I		Procedure II			
	mg. %	%	mg. %	%	mg. %	%
Whole homogenate	70.6 ±8.5	—	49.0 ±8.7	—	250.0 ±31.1	—
Nuclear-mitochondrial fraction	49.0 ±7.5	68.2	37.0 ±7.6	71.1	89.0 ±18.6	31.9
Microsomal-cytoplasmic fraction	22.8 ±4.6	31.8	15.0 ±4.1	28.9	190.0 ±41.1	68.1
Sum	71.8	100.0	52.0	100.0	279.0	100.0

The amount of sialic acid found in the homogenates purified by tannin was higher than in those treated with sulphosalicylic acid, the difference being statistically significant. The lost 20 mg. of sialic acid could not be found in the sulphosalicylic acid-soluble proteins reacting with tannin.

In the two subcellular fractions studied after purification with sulphosalicylic acid the average values for sialic acid content were also lower than after purification with tannin, but the differences were not statistically significant.

DISCUSSION

The orcinol, resorcinol and diphenylamine tests are not specific for sialic acid and before carrying out the estimations in tissue hydrolysates, interfering substances

must be eliminated by ion exchangers. Without this purification, 2 - 10 fold higher values, depending on the kind of tissue, are obtained. Warren (1959) using thiobarbituric acid obtained without the ion-exchange procedure the same results as by the resorcinol method of Svennerholm (1958) in which the purification on Dowex-1-acetate is applied. In the method of Warren the interfering effect of deoxyribose is eliminated by extraction with cyclohexanone of the coloured compound derived from sialic acid and by reading the extinction at 549 mμ. The complex formed from deoxyribose is scarcely soluble in cyclohexanone and has an absorption maximum at 532 mμ.

Another thiobarbituric acid method was presented by Aminoff (1961) and it also allows a direct determination of sialic acid. No colour reaction is given by fructose (0.2 mg. per sample), sucrose (0.1 mg.), ribose-5-phosphate (0.1 mg.) and aminosugars (1 mg.).

So far these methods have not been applied to sucrose homogenates because high concentrations of sucrose interfere both with the thiobarbituric acid methods and with the diphenylamine and resorcinol methods. The elaborated procedures for purification of homogenates and subcellular fractions from sucrose and non-protein compounds by tannin or sulphosalicylic acid, appeared to be suitable for preparation of the material for determination of sialic acid either by the specific thiobarbituric acid method, or by the much less specific diphenylamine method. The determinations of sialic acid made by the two methods were in agreement which indicates that the obtained sialoprotein fractions were free of interfering compounds. In the procedure applied, DNA and RNA were removed from the nuclear-mitochondrial fraction by washing several times with an aqueous solution of tannin or sulphosalicylic acid.

The concentration of sialic acid in rabbit kidney determined in hydrolysates amounted to 70 mg. per 100 g. fresh weight, being the same as the concentration of *N*-acetylneuraminic acid in blood serum and twice as high as that reported by Svennerholm (cited after Gottschalk, 1960) in the kidney of man (35.5 mg.%), ox (29.9 mg.%), calf (48.7 mg.%) and hog (33.5 mg.%). No data concerning the content of sialic acid in rabbit kidney were found in the literature.

Kidney sialoproteins, in contrast to blood serum proteins, do not form a sulphosalicylic acid-soluble fraction. Sialic acid was found only in the protein insoluble in sulphosalicylic acid, and mainly in the nuclear-mitochondrial fraction. This fraction contained one-third of the total sulphosalicylic acid-soluble protein of a basic character, which originated from mitochondria as the nuclear histones are insoluble in 0.15 M-sulphosalicylic acid. Brunngraber & Brown (1963, 1964) demonstrated the presence of sialomucopolysaccharides in mitochondria and microsomes of dog brain, and it seems possible to suppose that these compounds are present also in rabbit kidney mitochondria. Very probably the acid mucopolysaccharides or sialoproteins form a ionic counterpart of the basic protein isolated by Rzeczycki *et al.* (1962) from kidney and liver mitochondria. The occurrence of sialomucopolysaccharides easily dissociating from the protein seems to be supported by the observed difference in

the amount of sialic acid in kidney homogenate after tannin and sulphosalicylic acid procedures. The homogenate purified by tannin contained on the average 70 mg.% of sialic acid, and the homogenate purified with sulphosalicylic acid, 50 mg.%. The lacking 20 mg.% of sialic acid may form part of sialomucopolysaccharide which dissociated from basic protein, passed to the sulphosalicylic acid solution, and, not being bound to protein, did not react with tannin. Another explanation may be the assumption of a very labile fraction containing sialic acid, which could be hydrolysed by 0.15 M-sulphosalicylic acid already at room temperature and be removed during washing of the sediment.

REFERENCES

Aminoff D. (1961). *Biochem. J.* **81**, 384.
Armitage R., Bayliss G.S., Gramshaw J.W., Haslam E., Haworth R.D., Jones K., Rogers H.J. & Searle T. (1961). *J. Chem. Soc.* 1842.
Brunngraber E.G. & Brown B.D. (1963). *Biochim. Biophys. Acta* **69**, 581.
Brunngraber E.G. & Brown B.D. (1964). *Biochim. Biophys. Acta* **83**, 357.
Gottschalk A. (1960). *The Chemistry and Biology of Sialic Acids and Related Substances.* Cambridge University Press.
Mejbaum-Katzenellenbogen W. (1955). *Acta Biochim. Polon.* **2**, 279.
Mejbaum-Katzenellenbogen W. & Wieczorek E. (1966). *Acta Biochim. Polon.* **13**, 69.
Rzeczycki W., Grudzińska A. & Hillar M. (1962). *Acta Biochim. Polon.* **9**, 295.
Seifer A. & Gerstenfeld S. (1955). *J. Lab. Clin. Med.* **50**, 17.
Svennerholm G.L. (1958). *Acta Chem. Scand.* **12**, 547.
Warren L. (1959). *J. Biol. Chem.* **234**, 1971.

KWAS SJALOWY W NERKACH KRÓLIKA

Streszczenie

1. Opracowano dwa sposoby (z zastosowaniem taniny i kwasu sulfosalicylowego) usuwania substancji interferujących w oznaczaniu kwasu sjalowego.

2. Stwierdzono, że nerki królika zawierają 70 mg.% kwasu sjalowego, głównie we frakcji jądrowo-mitochondrialnej.

Received 25 July, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

R. KĘDRACKI and W. SZER

A NOTE ON THE EFFECT OF SPERMINE ON DEGRADATION OF PYRIMIDINE POLYNUCLEOTIDES BY PANCREATIC RIBONUCLEASE

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

1. The rate of hydrolysis of high molecular substrates of ribonuclease decreases in the presence of spermine; the rate of hydrolysis of low molecular substrates remains unchanged. 2. The attainment of an ordered state by poly-uridylic acid in the presence of spermine does not prevent, in a specific way, its degradation by ribonuclease. 3. It is concluded that the effect of spermine on ribonuclease action should be attributed to the general ability of polyamines to bind polyribonucleotide phosphate. Enzymic protonation of high molecular substrates which determines the first step of enzyme action, is presumably hindered.

Recent experiments from this laboratory have shown that polyamines enhance substantially the formation and thermal stability of the ordered state of synthetic polyribonucleotides capable of undergoing a co-operative transition (Szer, 1966 a,b); e.g. poly-U[1] in the presence of spermine attains an ordered state with 45% temperature-dependent hyperchromicity and undergoes an abrupt transition at 29.4°. Thus, poly-U becomes a suitable model for, amongst others, investigations on the impact of secondary structure on endonuclease action. The melting behaviour of poly-C, which is known to possess a broad melting profile at neutral pH with no indication of co-operativeness, was not affected by spermine. On the other hand, conductometric studies have shown that spermine possesses high affinity for any polynucleotide, and neutralizes effectively its phosphate backbone charge; when added in excess to any synthetic homopolyribonucleotide, a precipitate forms instantaneously (Felsenfeld, 1962). These differences and analogies in behaviour of poly-C and poly-U prompted us to examine the susceptibility of both polypyrimidines to RNase in the presence of spermine. Several investigators have shown that natural RNA's become less susceptible to the action of endonucleases in the presence of spermine, presumably due to configurational changes brought about

[1] Abbreviations: poly-U, poly-uridylic acid; poly-rT, poly-ribothymidylic acid; poly-C, poly-cytidylic acid; poly-A, poly-adenylic acid; poly-(A+U), twin-stranded complex of poly-U and poly-A. Other abbreviations are used according to Tentative Rules of IUPAC-IUB, 1965, *J. Biol. Chem.* **241**, 527.

by the polyamine. These changes are assumed to make the RNA's more compact, better prevented from temperature-induced unfolding and, hence, less penetrable to enzymes (Herbst & Doctor, 1959; Mitra & Kaesberg, 1963; Amos & Kearns, 1963).

MATERIALS AND METHODS

Poly-U, poly-C and poly-A were commercial products (CalBiochem, Los Angeles, California, U.S.A.) purified prior to use by deproteinization and exhaustive dialysis, finally against distilled water. Yeast transfer RNA was prepared according to von Ehrenstein & Lipmann (1961). Middle silk gland RNA (*Bombyx mori* L.) was kindly provided by Mrs. J. Passent from this Institute and UpU was a generous gift from Dr. H. Witzel, Institute of Chemistry, Marburg University, Germany. 2′:3′-Cyclic phosphates of uridine, cytidine and adenosine were prepared as elsewhere described (Szer & Shugar, 1963). Pancreatic ribonuclease (Light Co., Colnbrook, England), four times recrystallized was used throughout. Unicam SP 500 conventional and SP 800 self-recording UV spectrophotometers were employed. The increase in extinction at λ max. was taken as a measure of enzymic hydrolysis of substrate. Substrates were prepared at a concentration of 5×10^{-5} M to 1×10^{-4} M in 0.01 M-phosphate buffer, pH 7.4, and incubated at 18° directly in thermostated spectral cuvettes. One equivalent spermine per mole of nucleotide phosphorus was added. Enzyme concentrations are given in legends to figures. In some instances, as indicated in the text, hydrolysis of substrates was followed by paper chromatography. Substrates were then made up at 10^{-2} M in 0.05 M-phosphate buffer, pH 7.4. Incubation was at 18° and samples were withdrawn at various time intervals for ascending chromatography in ethanol - 1 M-ammonium acetate (5 : 2, v/v) system on Whatman no. 1 paper.

RESULTS AND DISCUSSION

There is a small but definite decrease in the rate of hydrolysis of natural RNA's by RNase in the presence of spermine. This was less pronounced with the high molecular silk gland RNA than with yeast transfer RNA (Fig. 1).

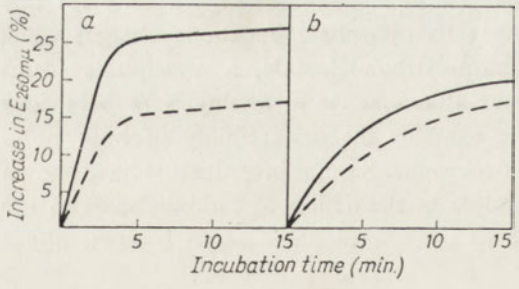


Fig. 1. Hydrolysis of natural RNA by pancreatic ribonuclease. Incubation at 18°, enzyme concentration 0.04 μg./ml. (———), Control without spermine; (— — —), with spermine; (*a*), yeast transfer RNA; (*b*), silk gland RNA.

The degradation of poly-U is more complex and proceeds in two clearly distinguishable steps. The first step is consistent with a transition from the ordered state to the random coil and is characterized by the disappearance of structural

hyperchromicity (Fig. 2, transition from curve *A* to *B*). The second one represents hydrolysis to mononucleotides and a gradual loss of residual hyperchromicity is observed (Fig. 2, transition from curve *B* to *C*). Obviously, on hydrolysis of the random coil in the absence of spermine only the loss of residual hyperchromicity is

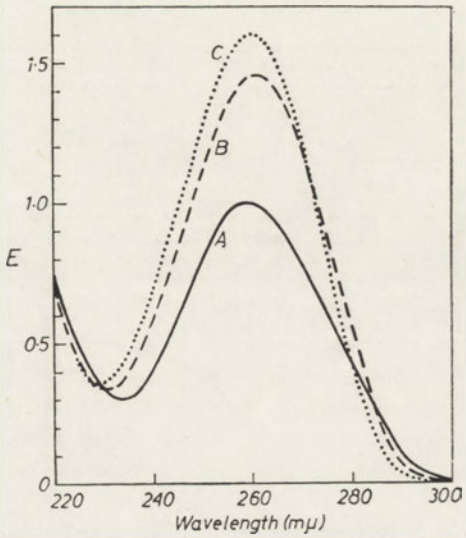


Fig. 2. Absorption spectrum of poly-U in 0.01 M-phosphate buffer, pH 7, at polymer phosphate : equivalent spermine ratio of 1 : 1. Curve *A*, at 18°; curve *B*, at 40°; curve *C*, mononucleotides, after enzymic hydrolysis.

observed. The latter step is followed by the opening of cyclic phosphates; however, due to small enzyme concentration it proceeds rather slowly and will not be taken into account. Contrary to what might have been expected, the presence of the ordered state in poly-U does not prevent the degradation of the polymer by RNase and, moreover, the first step, accompanied by the large increase in extinction, proceeds rapidly (Fig. 3a). Indeed, at 3 μg./ml. enzyme the total structural hyperchromicity is lost within 0.5 min. while it takes 20 min. to bring about a 50% drop in residual hyperchromicity (Fig. 3a and 3b). In controls without spermine (Fig. 3c) comparable to the second step of the former experiment, the half time of degradation of the random coil is 4 min. i.e. it proceeds faster than with spermine, similarly to the results obtained with natural substrates. At 0.02 μg./ml. enzyme the ordered state disappears within 25 min. At this point the reaction mixture exhibits a profile identical with the random coil (Fig. 2, curve *B*) and it takes several hours to observe the degradation to monomers. Polyribothymidylic acid is known not to require spermine to form an ordered state with a temperature transition above room temperature since it "melts" at 34° in 10^{-3} M-Mg^{2+}. Under these circumstances it behaves similarly, i.e. a rapid increase in extinction consistent with the disappearance of the helical form (Szer, Świerkowski & Shugar, 1963) is followed by a gradual loss of residual hyperchromicity. Several possibilities were entertained to explain the rapid rate of the first step as compared with the gradual, spermine-inhibited second step. It cannot be *a priori* excluded that a basic protein like RNase with

many $-NH_3^+$ charged groups irregularly situated on its surface may compete with spermine for poly-U, or poly-rT, binding sites and disrupt the ordered state. Were this the case, one would expect to observe similar effects with other basic

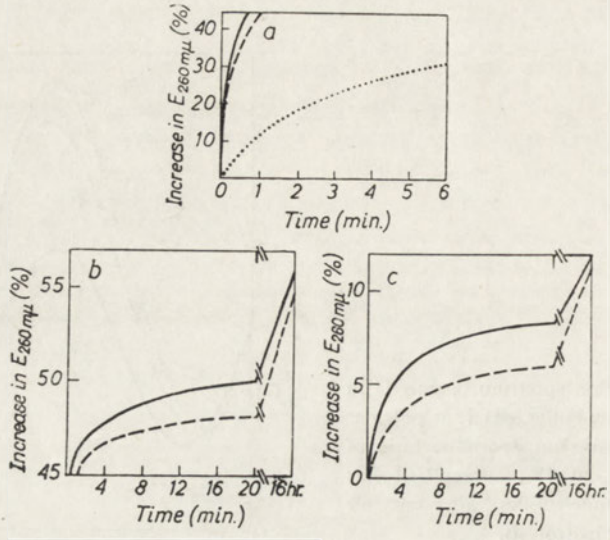


Fig. 3. Hydrolysis of poly-U by pancreatic ribonuclease. Incubation at 18°. Enzyme concentration: (———), 3 μg./ml.; (— — —), 1 μg./ml.; (• • • • •), 0.02 μg./ml.; (*a*), in the presence of spermine; (*b*), in the presence of spermine, continued; note different scale as compared to former curve; (*c*), control without spermine.

proteins, e.g. deoxyribonuclease. This possibility was checked with electrophoretically purified, RNase-free deoxyribonuclease (Worthington Co., Freehold, New Jersey, U.S.A.) and it was found that even at high enzyme concentration (10 μg./ml.) the

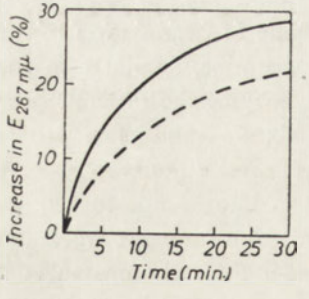


Fig. 4. Hydrolysis of poly-C by pancreatic ribonuclease. Incubation at 18°. Enzyme concentration 0.04 μg./ml. (———), Control without spermine; (— — —), with spermine.

ordered state of poly-U was not affected on prolonged treatment. We are therefore tempted to assume that occasional breaks in the poly-U chains caused by the enzyme produce fragments still exhibiting residual hyperchromicity but rendered uncapable of retaining the ordered state. We have observed that poly-U preparations exposed

for 10 min. to 0.1 N-KOH lost most of their structural hyperchromicity (a decrease from 45% to 12%) but none of their residual hyperchromicity. Such alkali-treated preparations did not produce small oligonucleotides as revealed by paper chromatography. It should be noted that such a behaviour of ordered poly-U toward RNase is not consonant with its behaviour in the poly-(A+U) complex in which it is much more resistant to RNase. In the former case all strands are simultaneously degraded, while in the latter only one strand is being fragmented and the stability of the duplex does not diminish until very small oligonucleotides are produced (Lipsett, Heppel & Bradley, 1961).

Figure 4 reveals that hydrolysis of poly-C by RNase is slowed down in the presence of spermine to an extent similar to that noted for natural substrates or for degradation of the random-coil form of poly-U. It appears that the effect of spermine on poly-U and poly-C, while quite different from the structural point, is similar in respect to hydrolysis by RNase. It is therefore concluded that the observed inhibition of RNase should be attributed to the ability of spermine to bind strongly polymer phosphate rather than to structural changes related to base-base interaction, and induced selectively by spermine toward poly-U but not toward poly-C. The polyamine may be thought to interfere with the enzyme at the substrate binding site and apparently render the phosphate groups less accessible to enzymic protonation. This, in turn, constitutes the first step in enzyme action (Witzel, 1963).

Further support for this notion was derived from experiments with a monoanionic diester UpU and with the 2′:3′-cyclic phosphates of uridine and cytidine. Since the affinity of spermine to phosphate residues is connected with the polyanionic nature of polynucleotides, one would not expect any particular influence of the agent upon the rate of hydrolysis of monomeric RNase substrates. This indeed was found to be the case with the cleavage of phosphodiester bond in UpU and with the internal diester bonds in 2′:3′-Up and 2′:3′-Cp.

Curiously enough enzymic tests performed at high substrate concentration (10^{-2} M) revealed an opposite effect of spermine toward both polypyrimidines. Degradation was more rapid with than without spermine. Conditions were found in which in the presence of spermine both polymers were affected to such an extent that movable fragments were detected by paper chromatography (18°, 15 min., 0.6 μg/ml. enzyme for poly-U and 0.85 μg/ml. enzyme for poly-C) while controls without spermine remained at the origin. Under these conditions the presence of spermine enhanced also hydrolysis of poly-A by the enzyme (Beers, 1960). It should be recalled, however, that in this range of homopolymer concentration there is a possibility of artifacts arising from intermolecular aggregation. Aggregates are slowly attacked by the enzyme, while the presence of spermine prevents aggregation through interaction with polymer phosphate. The latter effect might be more important for the rate of hydrolysis in concentrated solutions than the observed inhibition of enzyme action due to spermine interference at the substrate binding site.

The authors are indebted to Dr. H. Witzel for helpful discussions. This investigation has been aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research.

REFERENCES

Amos H. & Kearns A.E. (1963). *Exptl. Cell Res.* **32**, 14.
Beers R.F., Jr. (1960). *J. Biol. Chem.* **235**, 2393.
Von Ehrenstein G. & Lipmann F. (1961). *Proc. Natl. Acad. Sci. U.S.* **47**, 497.
Felsenfeld G. (1962). In *The Molecular Basis of Neoplasia*, p. 104. University of Texas Press, Austin.
Herbst E.J. & Doctor B.B. (1959). *J. Biol. Chem.* **234**, 1497.
Lipsett M.N., Heppel L.A. & Bradley D.F. (1961). *J. Biol. Chem.* **236**, 857.
Mitra S. & Kaesberg P. (1963). *Biochem. Biophys. Res. Commun.* **11**, 146.
Szer W. (1966a). *Acta Biochim. Polon.* **13**, 251.
Szer W. (1966b). *J. Mol. Biol.* **16**, 585.
Szer W. & Shugar D. (1963). In *Biochemical Preparations* (G.B. Brown, ed.) **10**, 139. J. Wiley & Sons, New York.
Szer W., Świerkowski M. & Shugar D. (1963). *Acta Biochim. Polon.* **10**, 87.
Witzel H. (1963). In *Progress in Nucleic Acid Research* (J.N. Davidson & W.E. Cohn, eds.) **2**, 221. Academic Press, New York & London.

UWAGI O WPŁYWIE SPERMINY NA ROZKŁAD POLIRYBONUKLEOTYDÓW PIRYMIDYNOWYCH DZIAŁANIEM RYBONUKLEAZY TRZUSTKOWEJ

Streszczenie

1. Szybkość hydrolizy wielkocząsteczkowych substratów rybonukleazy maleje w obecności sperminy; szybkość hydrolizy niskocząsteczkowych substratów nie zmienia się.

2. Przejście kwasu poliurydylowego w stan uporządkowany pod wpływem sperminy nie chroni go w specyficzny sposób przed działaniem rybonukleazy.

3. Wyrażono pogląd, że wpływ sperminy na działanie rybonukleazy trzustkowej jest związany z ogólną zdolnością poliamin do współdziałania z grupami fosforanowymi polinukleotydów. Utrudnia to enzymatyczną protonację wielkocząsteczkowego substratu, która stanowi pierwszy etap działania enzymu.

Received 19 September, 1966.

Note added in proof: Since the submission of the above manuscript we have found that the presence of spermine is without effect on the rate of hydrolysis of several natural RNA's and synthetic polyribonucleotides by an exonuclease, snake venom phosphodiesterase.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

IRENA PIETRZYKOWSKA and D. SHUGAR

STUDIES ON BACTERIOPHAGE AND BACTERIOPHAGE DNA CONTAINING 5-ETHYLURACIL OR 5-BROMOURACIL IN PLACE OF THYMINE

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa 12

1. Synthesis of bacteriophages T3 and T7, with thymine-dependent *E. coli* CR-34 as host cell, proceeds readily in the presence of the thymidine analogue, 5-ethyldeoxyuridine; under the same conditions 5-ethyluracil is much less effective. Under conditions where thymidylate synthetase is inhibited by 5-fluorodeoxyuridine, phages T2 and T4 can multiply only to a small extent in the presence of 5-ethyldeoxyuridine, but readily do so in the presence of either 5-bromouracil or 5-bromodeoxyuridine. By contrast phage T3 synthesis can be maintained in the presence of either 5-ethyldeoxyuridine or 5-bromodeoxyuridine. Phages T3 and T7, in which 66% of the thymine residues have been replaced by 5-ethyluracil, or 60% replaced by 5-bromouracil, retain their infectivity to the same degree as normal phage. 2. Isolated DNA from phage T3, with 66% of the thymine residues replaced by 5-ethyluracil, exhibits a T_m value which is 4.5° lower than that of normal T3 DNA, and which is independent of the salt concentration over the range 0.015 to 0.15 M. 3. On the other hand, phage T3 DNA, with 60% of the thymine residues replaced by 5-bromouracil, exhibits a higher stability than normal DNA at low salt concentrations (0.015 M). At higher salt concentrations, 0.15 M, this difference in stability, as estimated from the T_m value, disappears in agreement with observations on synthetic polynucleotide analogues. 4. Under optimal conditions at 65°, all the DNA samples renature completely, the rate for EtU-DNA and BrU-DNA being slightly less than that of the normal DNA.

It has previously been shown (Piechowska & Shugar, 1965) that 5-ethyluracil competitively antagonizes the utilization of thymine in cultures of thymine-dependent *E. coli* strains, and is capable of incorporation into the bacterial DNA to a moderate extent, about 15 - 18 mole%. The subsequent synthesis of EtUdR[1] (M. Świerkowski & D. Shugar, in preparation) suggested the possibility of incorporating this analogue in bacteriophage DNA, with a view to examining the resultant effects on both the intact phage and its isolated DNA. This was expected to be all the more interesting in view of the known modification of DNA stability by incorporated BrU (Kit &

[1] The following abbreviations are used in this text: BrU, 5-bromouracil; BrUdR, 5-bromodeoxyuridine; EtU, 5-ethyluracil; EtUdR, 5-ethyldeoxyuridine; TdR, thymidine; FUdR, 5-fluorodeoxyuridine; SSC, aqueous solution of 0.15 M-NaCl and 0.015 M-Na citrate; DSC, 0.015 M-NaCl and 0.0015 M-Na citrate; DNase, deoxyribonuclease; RNase, ribonuclease.

Hsu, 1961; Szybalski & Mennigman, 1962); as well as the previous demonstration that replacement of uracil residues by thymine in synthetic polynucleotides results in a marked enhancement of helix stability (Szer, Świerkowski & Shugar, 1963); this latter finding is in agreement with the fact that uracil-containing phage PBS2 DNA melts at a T_m 5° below that for the corresponding thymine-containing DNA with the same G-C content (Cassidy, Kahan & Alegria, 1965).

For purposes of comparison, phage DNA was also prepared with incorporated BrU. The replacement of thymine by 5-bromouracil in DNA is known to result in lethality and/or mutagenicity. The latter effect is generally presumed to be due to the ability of some of the BrU residues to occasionally pair with cytosine, although no direct evidence to this effect is extant. On the other hand, it has been shown that extensive replacement of thymine by BrU in transforming DNA does not affect the transforming activity (Szybalski *et al.*, 1960), although Gimlin, Farquarson & Leach (1963) have reported that high concentrations of BrU-containing *B. subtilis* transforming DNA exhibit a lethal effect on cells which are not transformed.[2]

Although BrU, or BrUdR, readily replaces thymine or thymidine in the DNA of a variety of bacteria and bacteriophages (Zamenhof & Griboff, 1954; Brockman & Anderson, 1963), its properties are markedly different from those of thymine, due to the strongly electronegative substituent, as a result of which the pK_a of the pyrimidine ring in the nucleoside or nucleotide is reduced from about 9.3 to 7.9 (Berens & Shugar, 1963). However, a 5-ethyl substituent in the pyrimidine ring only slightly modifies the pK_a (M. Świerkowski & D. Shugar, in preparation), and it is most unlikely that EtU possesses base-pairing properties different from those of either thymine or uracil.

MATERIALS AND METHODS

The coliphages employed in these studies were T2, T4, T3 and T7; and the host strains were *E. coli* B and the thymine-dependent *E. coli* CR-34.

Thymine, uracil, thymidine, bromouracil and bromodeoxyuridine were Sigma and CalBiochem products. The preparation of 5-ethyluracil and 5-ethyldeoxyuridine will be elsewhere described (M. Świerkowski & D. Shugar, in preparation). Most of the experiments were performed with a preparation of EtUdR containing an admixture of the α anomer, after it had been shown that the latter was without effect on phage multiplication by the use of crystalline samples of both the α and β anomers.

Pancreatic RNase was a Worthington crystalline preparation. Commercial streptodornase (Warsaw Serum and Vaccine Plant) was used as a source of DNase.

[2] Using a streptococcal system with a very high transforming efficiency, more than 60% for two non-linked markers, we have found that competent cells may be killed with homologous and heterologous *normal* DNA (Piechowska, Żiżina & Shugar, 1966). These results will be reported in detail elsewhere (M. Piechowska & D. Shugar, 1967).

Phage multiplication and attempts at incorporation of EtUdR were conducted on a glycerol medium (Fraser & Jerrel, 1953) to which either thymine or the required thymine analogue was added to the appropriate concentration.

Preparation of phage T3 containing EtUdR. Phage multiplication on the CR-34 host strain was carried out on the glycerol medium in the following manner: thymine was added to 2 liters medium to a final concentration of 10 μg./ml., and the medium inoculated with cells from two agar slants, so that the initial optical density of the culture was 0.03. Incubation was carried out at a temperature of 37° for 3 hr. to an optical density of 0.26. The cells were collected by centrifugation and washed twice with thymine-free glycerol medium. The resulting pellet was suspended in a few ml. medium and added to 2 liters of fresh preheated glycerol medium containing EtUdR at a concentration of 20 μg./ml. The optical density of the bacterial suspension was 0.28. After 10 min. incubation on a shaker, the suspension was infected with T3 phage at an infection multiplicity of 0.01, adsorption allowed to proceed for 5 min. without shaking, and incubation then conducted with shaking at 37° for 75 min., at which time all the cells had been lysed. To the lysate was added 20 mg. RNase (10 μg./ml.), incubation continued for 30 min. at 37°, and two vials of streptococcal DNase (25 000 international units each) then added, followed by a further 30 min. incubation. The lysate was stored overnight in the cold room and then filtered through a 5-mm. layer of Celite.

It should be noted that phages T3 and T7 synthesized in the presence of EtUdR largely retain their infectivity. This follows from a comparison of the phage yields in the presence of thymine or EtUdR. The yields are the same for T7 and only 1/3 less for EtUdR in the case of T3. In the latter instance this is most likely due to a smaller burst size; and this is supported by the fact that the yield of isolated DNA in the case of T3 was the same for phage cultivated in the presence of EtUdR as for the control, based on the amount of DNA per viable phage particle. The same conclusions were found to apply in the case of T3 cultivated in the presence of BrUdR.

Phage purification and DNA isolation. The phage filtrate was purified and concentrated by adsorption on hydroxyapatite as described by Miyazawa & Thomas (1965). The filtrate was diluted with phosphate buffer to a total volume of 7.4 liters so that the concentration of buffer was 0.02 M, pH 6.8, and to this was added 140 ml. of a thick suspension of hydroxyapatite. Adsorption was allowed to proceed for 30 min. at room temperature with gentle stirring. The fluid was then decanted and the precipitate suspended in 250 ml. of 0.02 M-phosphate buffer with added tris, pH 6.8, according to Miyazawa & Thomas (1965). The suspension was then poured into a 4-cm. diameter tube to form a column, which was eluted with 0.02 M buffer until the eluate exhibited an optical density of 0.09 at 260 mμ. The phage was then eluted with 0.1 M-phosphate buffer, pH 6.8, at a flow-rate of 0.5 ml. per min., portions of 3 ml. being collected to determine the phage titre and optical density at 260 mμ. The fraction with the high phage titre, 90 ml. in volume, was centrifuged for 4 hr. at 60 000 *g*. The pellets were taken up in 20 ml. of 0.5 M-NaCl containing 0.001

M-$MgCl_2$, left overnight in the cold and again centrifuged for 4 hr. at 60 000 *g*. The resulting pellet was taken up in 2 ml. salt solution as above, centrifuged at slow speed, and the DNA then extracted with freshly distilled phenol which had been neutralized with NaOH (0.05 ml. 10 N-NaOH per 10 ml. phenol) and saturated with the same salt solution used for the phage suspension. Following three extractions as described by Frankel (1965), the aqueous phase was exhaustively dialysed against

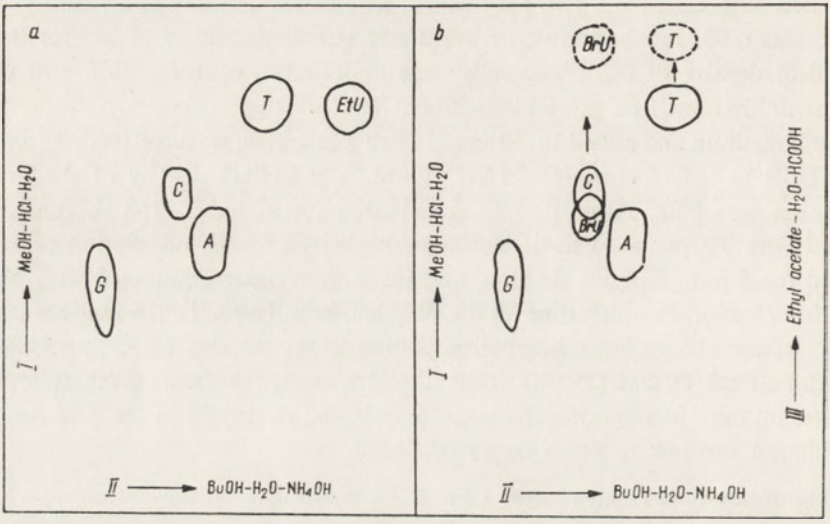


Fig. 1. Two-dimensional thin-layer chromatography of DNA hydrolysates: (*a*), showing resolution of thymine and 5-ethyluracil from each other and from guanine, adenine and cytosine; (*b*), showing how the use of an additional solvent (*III*) results in resolution of 5-bromouracil and thymine from each other and from the other bases.

8 portions of 2 liters DSC. The resulting solution of DNA was stored in the deep-freeze. Phage T3 containing incorporated BrUdR was prepared in an analogous manner. Phage T3 control DNA was prepared using *E. coli* B as the host cell.

The UV absorption spectra of the three DNA preparations exhibited $\varepsilon_{260}/\varepsilon_{230}$ values of 2.22 for the control, 2.26 for the EtU-DNA and 1.98 for the BrU-DNA; these are to be compared with a range of 1.8 - 2.2 given by Miyazawa & Thomas (1965) for different DNA samples. The corresponding $\varepsilon_{260}/\varepsilon_{280}$ values were 1.85, 1.86 and 1.80, as compared to the range of 1.6 - 2.0 given by Miyazawa & Thomas (1965).

Chromatography of phage T3 DNA hydrolysates. About 150 μg. of freeze-dried DNA was hydrolysed in a sealed tube with 80 μl. of conc. formic acid for 45 min. at 175°. The hydrolysates were subjected to thin-layer chromatography in two dimensions on MN-300-G cellulose according to Randerath (1965). Solvent *I* was methanol - conc. HCl - H_2O (70 : 20 : 10, by vol.), and solvent *II* butanol - water - 28% NH_4OH (87 : 8 : 5, by vol.). This combination clearly resolves thymine and 5-ethyluracil from each other and from the other bases (Fig. 1a). With the same solvent system, however, 5-bromouracil overlaps with cytosine, and a third solvent

system was employed, ethyl acetate - H_2O - HCOOH (60 : 35 : 5, by vol.), as shown in Fig. 1b to give a good separation of bromouracil from thymine and other bases. The bases were eluted with 0.02 N-HCl by the method of Randerath & Randerath (1965), after checking in several trial runs that elution carried out in this manner was quantitative. When the eluates had been taken up on paper strips, they were subsequently eluted into 0.02 N-HCl with warming to 60 - 70°, and then subjected to spectral analysis, against corresponding controls obtained from an area adjacent to the eluted base.

Melting profiles were obtained on a Unicam SP 500 spectrophotometer fitted with a special heating block in which circulated a glycerol-water mixture from a Hoeppler ultrathermostat. A thermistor in a dummy cuvette was used for temperature measurements, and 10 - 15 min. was allowed for equilibrium to be established at each temperature. The DNA solutions, about 20 μg./ml., were first centrifuged at 7 000 *g*, then dialysed against the appropriate solvent for 6 - 12 hr.

RESULTS

Phage synthesis in presence of EtUdR. Phage synthesis was examined in the presence of EtU and EtUdR, in place of thymine, using the thymine-dependent *E. coli* CR-34 strain as host. In view of the fact that the T-even phages are capable of inducing synthesis of thymidine in a thymine-dependent host, phage T3 was first employed since, along with phages T1 and T7, it does not possess this ability (Stent, 1963). Bacteria were cultivated on a glycerol medium, containing 10 μg./ml. thymine, to an optical density of 0.2 (1 - 2×10^8 cells/ml.), collected by centrifugation, washed twice with thymine-free medium, and suspended in freshly prewarmed thymine-free medium to give an optical density of 0.2. The suspension was divided into four portions in small flasks. To each of these was added, in turn, thymine and EtU to a concentration of 10 μg./ml., EtUdR to a concentration of 20 μg./ml., and an equal volume of water to the fourth flask to serve as control. All four flasks were incubated at 37° on a shaker for 10 min., and phage then added to all of them to give an infection multiplicity of 0.01. Following 5 min. without shaking to allow for phage adsorption, incubation was continued on a shaker and aliquots withdrawn at various time intervals for measurements of phage titre. The results, represented in Fig. 2, showed that EtUdR is capable of supporting the growth of T3 in the absence of thymine. In the sample containing EtUdR there is a more than 100-fold increase in the number of infective plaques as compared to the control without thymine, but slightly less than that in the presence of thymine, and one order of magnitude lower than in the presence of thymidine. It follows that EtUdR is capable of replacing thymine and thymidine in the reproduction of phage DNA. The figure also shows that EtU is much less effective, so that the host cells are unable to transform the base to the deoxyriboside to an appreciable degree, as already shown elsewhere (Piechowska & Shugar, 1965).

In view of the above results, it became of interest to determine whether EtUdR

could sustain the growth of other coliphages, in particular the T-even phages. Trials were therefore carried out with T2 and T4 under conditions where thymidylate synthetase was inhibited by FUdR, as normally applied for the incorporation into phage DNA of BrU or BrUdR (Koziński & Koziński, 1963). From Fig. 3 it will be seen that synthesis of phage T2 on *E. coli* CR-34 in the presence of EtUdR is appreciably less than for phage T3 under analogous conditions (Fig. 2b). However, under the same conditions, BrU and BrUdR are as effective as TdR in supporting synthesis of phage T2 (Koziński & Koziński, 1963). Phage T4 exhibited a similar behaviour. It is worth noting, in this connection, that phage T3, under conditions where FUdR is added to the medium to inhibit thymidylate synthetase, can utilize EtUdR for phage synthesis, albeit with a certain initial lag (Fig. 2b). Synthesis of phages T2 and T3 on *E. coli* B, in the presence of FUdR to inhibit thymidylate synthetase, does not occur in the presence of EtUdR.

Of the other odd phages, T7 was found to utilize EtUdR, with *E. coli* CR-34 as host, even more effectively than T3, the rate of synthesis being almost identical with that obtained in the presence of thymine or thymidine.

The foregoing results indicate appreciable incorporation of EtUdR into the DNA of phages T3 and T7. Phage T3 synthesis in the presence of EtUdR was therefore carried out on a larger scale, followed by isolation of the DNA as described

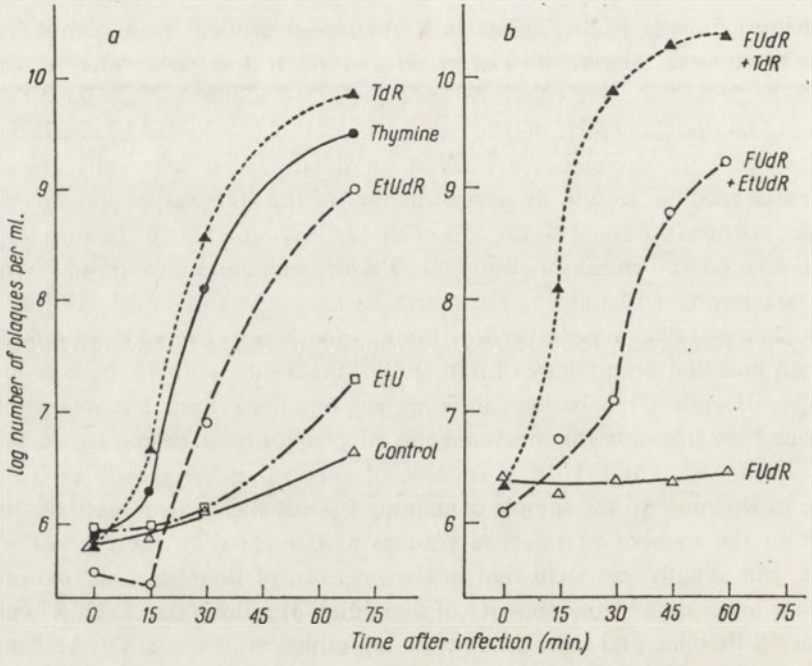


Fig. 2. Synthesis of phage T3 on *E. coli* CR-34: (*a*), in the presence of EtUdR (20 μg./ml.) and EtU (10 μg./ml.); and TdR (20 μg./ml.) and thymine (10 μg./ml.); (*b*), in the presence of EtUdR (20 μg./ml.) and TdR (20 μg./ml.) under conditions where thymidylate synthetase is inhibited by FUdR (5 μg./ml.). A control is shown with FUdR alone.

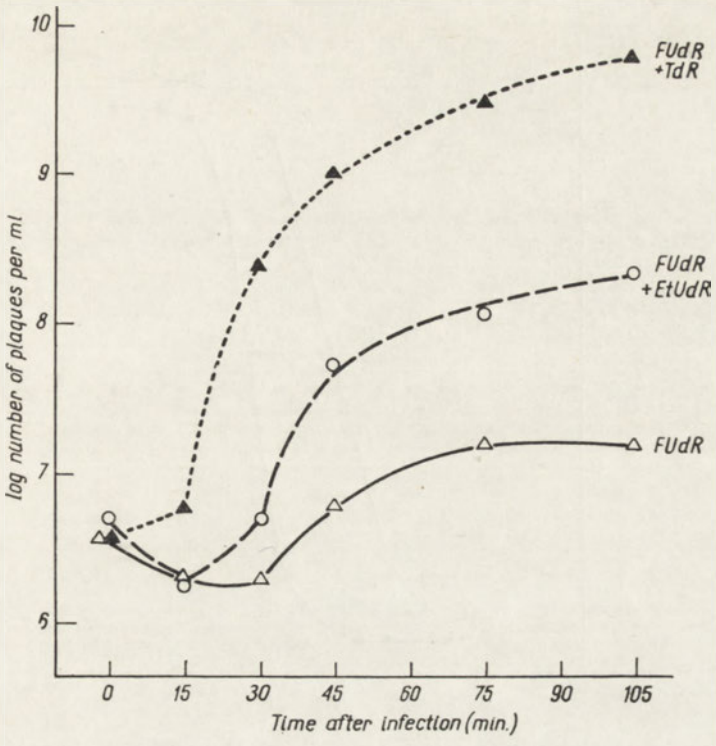


Fig. 3. Synthesis of phage T2 on *E. coli* CR-34 in the presence of EtUdR (20 μg./ml.) with FUdR-inhibition of thymidylate synthetase (FUdR, 5 μg./ml.), and under the same conditions with TdR (20 μg./ml.). A control is shown with FUdR alone.

in Materials and Methods. Hydrolysis of the resulting DNA, followed by chromatography and elution of the bases, as described above, showed that 66% of the thymine residues had been replaced by EtU. Under the same conditions, but using BrUdR in place of EtUdR, 61% of the thymine residues were replaced by BrU.

Temperature profiles of T3 DNA with incorporated EtUdR. Figure 4 exhibits the temperature profile, in DSC at neutral pH, of T3 DNA in which 66% of the thymine residues have been replaced by EtUdR, together with the profile of normal T3 DNA. It will be seen that the temperature hyperchromicity is almost identical for the two DNA samples, but that incorporation of EtUdR results in a decrease in stability, the resulting T_m being 4.4° lower than that of the control. The profile for the sample containing incorporated EtUdR is somewhat broader than that of the control. However, this apparent difference in breadth of the profiles disappears at higher salt concentrations. In Fig. 5 are shown profiles for the same samples at a tenfold higher salt concentration, in SSC. It will be noted that the difference in T_m values is unchanged under these conditions. Furthermore, at this higher salt concentration, which is favourable for renaturation, the cooling of both samples

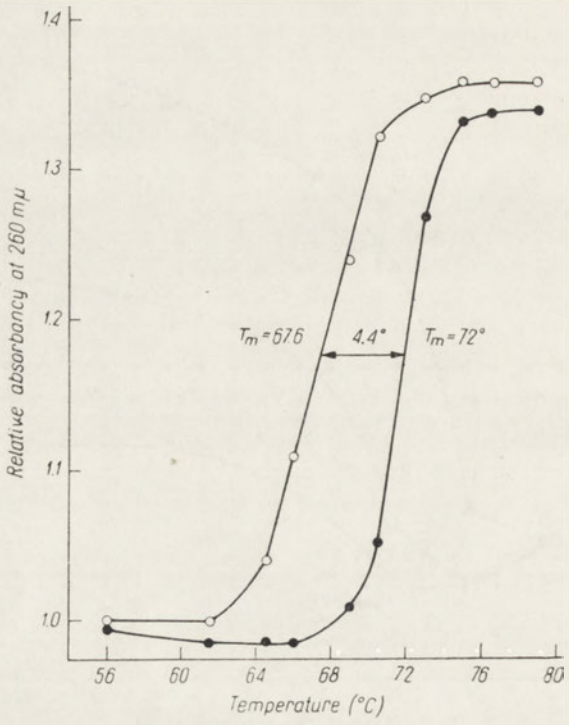


Fig. 4. Temperature profiles in DSC at neutral pH of: (●), phage T3 normal DNA; (○), phage T3 DNA in which 66% of the thymine residues have been replaced by 5-ethyluracil.

leads to practically complete renaturation, as shown by the fact that the initial hypochromicity is regained, and as is commonly observed for bacteriophage DNA (Marmur & Doty, 1961). Finally, when a second profile was run on each sample, following renaturation, the profiles were again almost identical, testifying to the completeness of renaturation during the cooling cycle. It should be noted that, during the cooling cycle, the EtUdR-DNA initially renatures more slowly than the control. However, when the kinetics of renaturation are followed under more optimal conditions, 65°, well below the melting temperatures, the differences in rates of renaturation between EtUdR-DNA, BrUdR-DNA and the control are considerably reduced (see below).

Temperature profiles of T3 DNA with incorporated BrUdR. In view of the foregoing findings, it appeared of interest to examine the influence of incorporated bromouracil on T3 DNA. From studies on synthetic polynucleotides, one would expect incorporation of BrUdR to enhance the stability of DNA (Inman & Baldwin, 1962), although the effect has been shown to be dependent on salt concentration (Inman & Baldwin, 1962). Several observers have already reported an enhanced stability of DNA in which thymine has been replaced by BrU (Kit & Hsu, 1961; Szybalski & Mennigmann, 1962).

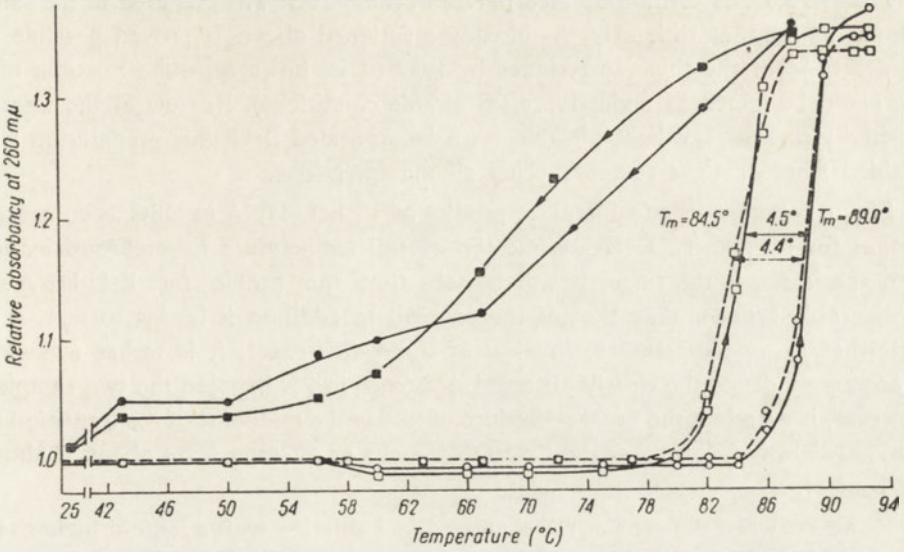


Fig. 5. Temperature profiles in SSC at neutral pH of normal phage T3 DNA and of DNA in which 66% thymine residues are replaced by EtUdR: (○—○), initial profile of normal DNA; (□—□), initial profile of EtUdR-DNA; (●—●), reverse (cooling) profile of normal DNA; (■—■), reverse (cooling) profile of EtUdR-DNA; (○— — —○), 2nd profile for normal DNA; (□— — —□), 2nd profile for EtUdR-DNA.

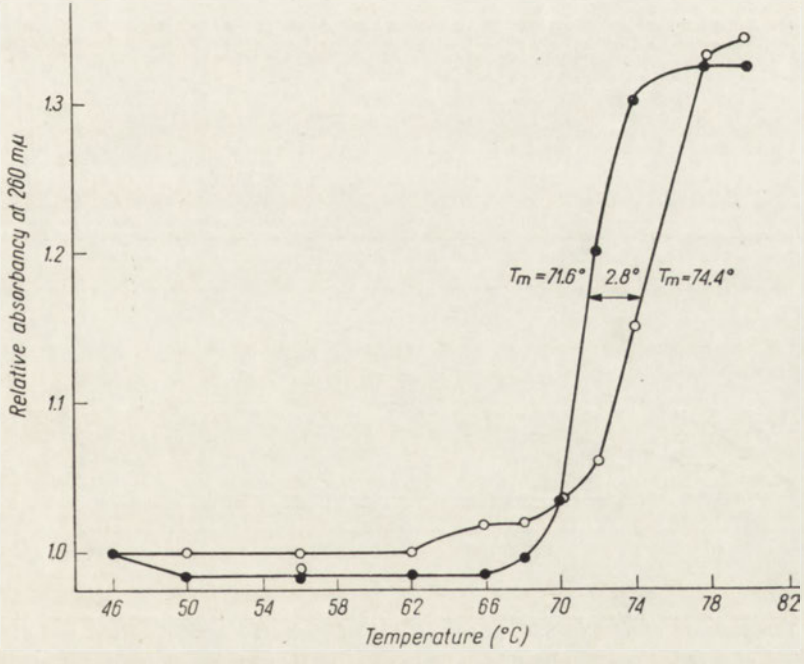


Fig. 6. Temperature profiles in DSC at neutral pH of: (●), phage T3 normal DNA; (○), phage T3 DNA in which 60% of thymine residues have been replaced by BrU.

Phage T3 DNA containing incorporated bromouracil was prepared in the same manner as for that with EtU. As already mentioned above, it proved possible to replace 61% of the thymine residues by BrU (it is, however, quite possible that this could be increased under more favourable conditions). In view of the known sensitivity to near UV light of DNA with incorporated BrU, this preparation was shielded from daylight sources during all manipulations.

Figure 6 exhibits the temperature profiles of T3 BrU-DNA in DSC as compared to that for normal T3 DNA. While the overall temperature hyperchromicity for both specimens is the same, it will be seen that the profile for BrU-DNA is considerably broader than that of the control. In addition it begins to melt at a somewhat lower temperature. In view of these differences, it is rather awkward to accurately define the clearly observed difference in T_m between the two samples. However, if we adopt the usual procedure of taking the midpoint of the transition as the T_m value, it follows that the BrU-DNA melts at a temperature about 3° above the control.

However, when the profiles are examined in a solution with a tenfold higher salt concentration, SSC, the difference in melting points practically disappears, with the exception that the BrU-DNA specimen exhibits some melting before the control. Here also it will be observed that, on cooling, renaturation is practically quantitative;

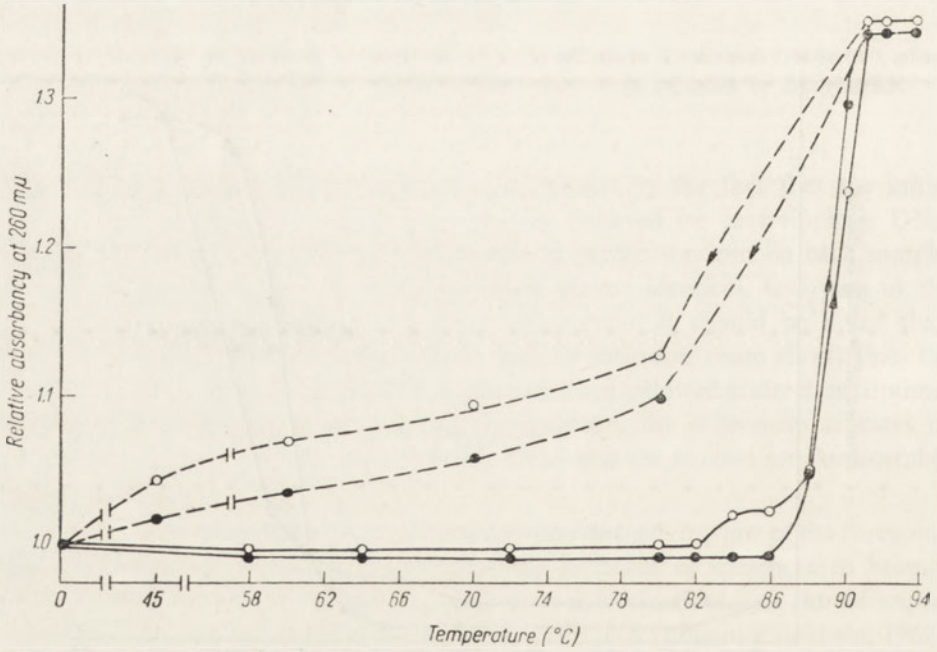


Fig. 7. Temperature profiles in SSC at neutral pH of phage T3 normal DNA, and of DNA in which 60% of thymine residues have been replaced by BrU: (●), initial profile for normal DNA (T_m = 89.0°); (○), initial profile for BrU-DNA (T_m = 89.3°); (●— — —●), reverse cooling profile for normal DNA; (○— — —○), reverse cooling profile for BrU-DNA.

and this is further substantiated by the fact that when a second profile was run on each sample, they were very close to those of the initial profiles. Furthermore, when the kinetics of renaturation of both samples were measured, by following the rates of decrease in optical density at 260 mμ, in SSC at 80°, the rate of renaturation of the BrU-DNA was found to be only slightly less than that of the control (Fig. 7).

In view of the foregoing, and the fact that at elevated temperatures EtU-DNA appears to renature more slowly than the normal, control, DNA, it appeared advisable to examine the kinetics of renaturation at a temperature farther removed

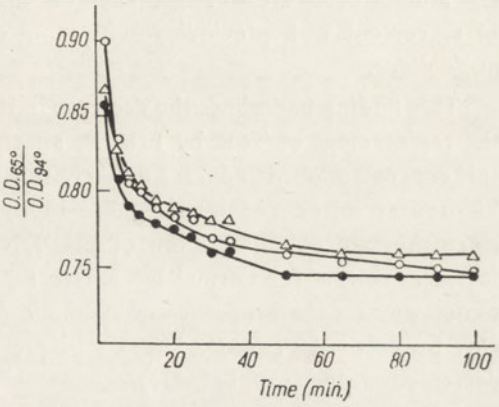


Fig. 8. Kinetics of renaturation in SSC at 65°, as followed spectrally by the decrease in optical density at 260 mμ, of: (●), phage T3 normal DNA; (○), phage T3 EtU-DNA; (△), phage T3 BrU-DNA.

from the melting points of all the DNA samples. The kinetics of renaturation were therefore examined under more optimal conditions for this reaction, i.e. 65°. The results are exhibited in Fig. 8, from which it will be seen that the differences between BrU-DNA and EtU-DNA on the one hand, and the control DNA on the other, are considerably reduced.

DISCUSSION

The extent of incorporation of EtUdR by phages T3 and T7, more than 60% of the thymine residues, is fairly high. It will be of interest to examine whether such incorporation is accompanied by mutagenesis as in the case of uptake of BrUdR.

It is pertinent to inquire as to why phages T2 and T4 are less capable of taking up this analogue in place of thymine or thymidine. This difference in behaviour is undoubtedly related to the different metabolic pathways of DNA synthesis following infection by the T-even and T-odd phages. It is possible that the triphosphate of EtUdR is not as effective a substrate for phage T2 DNA polymerase. Or it may be that the kinases induced following phage T2 infection are less active against EtUdR; it should be recalled that the T-even phages induce the synthesis of kinases which differ from those of the host cell (Cohen, 1963), whereas the T-odd phages utilize the kinases of the host cell (Cohen, 1963). Another possibility exists, viz. although EtUdR may be a substrate for the above-mentioned enzymes, the synthesis of phage

T2 DNA may be partially inhibited at the level of the DNA glucoside transferases, which are highly specific (Cohen, 1963). It is hoped to be able to test some of the foregoing possibilities by the preparation of the mono-, pyro- and triphosphates of EtUdR. It is of interest in this connection that BrUdR is as readily incorporated by T3 as T2. Furthermore, the fact that EtUdR does not support the synthesis of phages T2 and T3 on *E. coli* B, as compared to *E. coli* CR-34, shows that the ability to utilize EtUdR in DNA synthesis is dependent not only on the nature of the phage, but also the host.

The enhanced stability of phage T3 BrU-DNA at low salt concentrations, and the almost complete disappearance of this effect at higher salt concentrations, is in agreement with previous results reported on both synthetic and natural polynucleotides.

This finding underlines the decrease in stability of phage T3 DNA accompanying the replacement of TdR by EtUdR, an effect which is apparently independent of salt concentration. It appears unlikely that this decrease in stability is due to steric hindrances introduced by the more bulky ethyl (as compared to methyl group), since the rate of renaturation of EtU-DNA, under optimal conditions, is almost identical to that for normal DNA (see Fig. 8). It is hoped that investigations now under way on the properties of synthetic poly-EtU and its complexes with poly-A (M. Świerkowski and D. Shugar, in preparation) will help to account for the decreased stability of EtU-DNA.

We are indebted to Dr. M. Meselson of Harvard University for supplying the *E. coli* CR-34 strain, to the Cancer Chemotherapy National Service Center (USA) for a gift of 5-fluorodeoxyuridine, and to Mrs. M. Piechowska and Mr. M. Świerkowski for assistance and discussion. This investigation profited from the support of the World Health Organization and the Wellcome Trust.

REFERENCES

Berens K. & Shugar D. (1963). *Acta Biochim. Polon.* **10**, 25.
Brockman R.W. & Anderson E.P. (1963). *Pyrimidine Analogues in Metabolic Inhibitors* (Hochster R.M. & Quastel J.H., eds.) vol. 1, p. 261. Acad. Press, New York, London.
Cassidy P., Kahan F. & Alegria A.A. (1965). *Fed. Proc.* **24**, 226.
Cohen S.S. (1963). *Ann. Rev. Biochem.* **32**, 83.
Frankel F.R. (1963). *Proc. Natl. Acad. Sci. Wash.* **49**, 336.
Fraser D. & Jerrel E.A. (1953). *J. Biol. Chem.* **205**, 291.
Gimlin D.L., Farquarson E. & Leach F.E. (1963). *Nature* **197**, 407.
Inman R.B. & Baldwin R. (1962). *J. Mol. Biol.* **5**, 172, 185.
Kit S. & Hsu T.C. (1961). *Biochem. Biophys. Res. Commun.* **5**, 120.
Kozinski A.W. & Kozinski P.B. (1963). *Virology* **20**, 213.
Marmur J. & Doty P. (1961). *J. Mol. Biol.* **3**, 585.
Miyazawa Y. & Thomas C.A. Jr. (1965). *J. Mol. Biol.* **11**, 223.

Piechowska M. & Shugar D. (1965). *Biochem. Biophys. Res. Commun.* **20**, 768.
Piechowska M., Żiżina G. & Shugar D. (1966). *Symposium Isolierung und Struktur von DNA aus Mikroorganismen, Jena 5-9 Juli.*
Piechowska M. & Shugar D. (1967). *Acta Biochim. Polon.* **14**, no. 2.
Randerath K. (1965). *Nature* **205**, 908.
Randerath E. & Randerath K. (1965). *Anal. Biochem.* **12**, 83.
Stent G.S. (1963). In *Molecular Biology of Bacterial Viruses*, p. 149. Freeman W.H.& Company, San Francisco, London.
Szer W., Świerkowski M. & Shugar D. (1963). *Acta Biochim. Polon.* **10**, 87.
Szybalski W., Opara-Kubińska Z., Lorkiewicz Z., Ephrati-Elizur E. & Zamenhof S. (1960). *Nature* **188**, 743.
Szybalski W. & Mennigmann H.D. (1962). *Anal. Biochem.* **3**, 267.
Zamenhof S. & Griboff G. (1954). *Nature* **174**, 306.

BADANIE BAKTERIOFAGÓW I ICH DNA ZAWIERAJĄCYCH 5-ETYLOURACYL LUB 5-BROMOURACYL ZAMIAST TYMINY

Streszczenie

1. Uzyskano syntezę bakteriofagów T_3 i T_7 na szczepie tyminozależnym *E. coli* CR-34 w obecności analogu tymidyny 5-etylodezoksyurydyny. W tych samych warunkach 5-etylouracyl jest znacznie mniej wykorzystywany do syntezy faga. W warunkach zahamowania syntetazy tymidylowej przez 5-fluorodezoksyurydynę, fagi T_2 i T_4 jedynie w małym stopniu mogą namnażać się w obecności 5-etylodezoksyurydyny, mimo że w tych samych warunkach z łatwością wykorzystują 5-bromouracyl lub 5-bromodezoksyurydynę. Synteza fagów T_3 natomiast zachodzi zarówno w obecności 5-etylodezoksyurydyny jak i 5-bromodezoksyurydyny. Fagi T_3 i T_7, w których 66% tyminy zostało zastąpione przez 5-etylouracyl lub 60% tyminy przez 5-bromouracyl, zachowują infekcyjność w takim samym stopniu jak fagi normalne.

2. DNA izolowane z fagów T_3, w których 66% tyminy zostało zastąpione przez 5-etylouracyl wykazuje obniżenie T_m o 4,5° w porównaniu z normalnym DNA tych fagów. Różnica w T_m występuje niezależnie od stężenia soli w badanych granicach 0.015 - 0.15 M.

3. W odróżnieniu od DNA fagów T_3 zawierającego 5-etylouracyl, DNA fagów T_3, w których 60% tyminy zostało zastąpione przez 5-bromouracyl, wykazuje w niższych stężeniach soli większą trwałość struktury drugorzędowej niż DNA fagów normalnych, co wyraża się podwyższeniem T_m o 2.8° w porównaniu z kontrolą. W wyższych stężeniach soli (0.15 M) różnica w T_m DNA-BrU i DNA normalnego zanika, podobnie jak obserwowano w przypadku syntetycznych polinukleotydów poli-A-BrU.

4. Wszystkie próbki DNA renaturowały się całkowicie w warunkach optymalnych (65°) z tym, że szybkość renaturacji DNA-EtU i DNA-BrU jest nieco mniejsza niż DNA normalnego.

Received 23 September, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

R. Y. HSU and H. A. LARDY

PIGEON LIVER MALIC ENZYME

IV. PYRUVATE REDUCTASE ACTIVITY OF THE CRYSTALLINE ENZYME

Institute for Enzyme Research, University of Wisconsin, Madison 53706

This paper is dedicated to Professor Joseph Heller on the occasion of his 70th birthday

1. Crystalline pigeon liver malic enzyme was found to catalyse $NADPH_2$-specific, Mg^{2+}- or Mn^{2+}-activated reduction of pyruvate to form L-lactate and NADP. 2. The stoicheiometry of this reaction was demonstrated. 3. The rate of pyruvate reduction is approximately 1% of the activity for oxidative decarboxylation of L-malate.

In addition to catalysing the oxidative decarboxylation of L-malate (reaction 1), and the decarboxylation of oxalacetate (reaction 2), crystalline pigeon liver malic enzyme has been found to catalyse a slow but measurable NADP-specific, Mn^{2+}- or Mg^{2+}-dependent reduction of pyruvate to form L-lactate (reaction 3):

$$\text{L-Malate} + \text{NADP} \underset{}{\overset{Mn^{2+} \text{ or } Mg^{2+}}{\rightleftharpoons}} HCO_3^- + \text{pyruvate} + NADPH_2 \qquad (1)$$

$$\text{Oxalacetate} \xrightarrow[\text{pH 4.5 NADP}]{Mn^{2+} \text{ or } Mg^{2+}} \text{pyruvate} + HCO_3^- \qquad (2)$$

$$\text{Pyruvate} + NADPH_2 \xrightarrow{Mn^{2+} \text{ or } Mg^{2+}} \text{L-lactate} + \text{NADP} \qquad (3)$$

Table 1 shows the components required for $NADPH_2$ oxidation by the crystalline enzyme in the absence of bicarbonate where the reverse of reaction (1) is non-operative.

The non-metal dependent oxidation of $NADPH_2$ and $NADH_2$ could be accounted for by a trace ($< 0.01\%$) of lactate dehydrogenase contaminating the crystalline malic enzyme preparation. The rate of $NADPH_2$ (but not $NADH_2$) oxidation increased significantly in the presence of either Mg^{2+} or Mn^{2+}. Pyruvate is also required for this oxidation. The initial rate of $NADPH_2$ oxidation is linear and is proportional to protein concentration at two protein levels. A specific activity for the metal-activated $NADPH_2$ oxidation was calculated to be 0.34 μmoles $NADPH_2$ oxidized $\times$ min.$^{-1}$ $\times$ mg.$^{-1}$ protein at pH 7.0 and 25°, and is equivalent to 1.1% of the rate of reaction 1.

Table 1

Requirements for oxidation of reduced nicotinamide-adenine dinucleotides by crystalline malic enzyme

The cuvettes contained triethanolamine buffer, pH 7.0, 400 μmoles; $NADPH_2$, 0.64 μmole; K-pyruvate, 40 μmoles; 1% crystalline bovine albumin, 1 mg.; $MgCl_2$, 24 μmoles; crystalline malic enzyme, 150 μg.; and water to make a total volume of 3.0 ml. Initial reaction rates were recorded at 340 mμ on a Cary Model 11 recording spectrophotometer. Temperature 25°. Triethanolamine (Fisher Scientific Company); $NADPH_2$, $NADH_2$ (P-L Biochemicals, Inc.); NAD (Sigma Chemical Company); potassium pyruvate, glycine (Calbiochem); crystalline bovine serum albumin (Armour Pharmaceutical Company); and crystalline muscle lactate dehydrogenase (Boehringer and Sohne) were purchased from the designated sources. Crystalline pigeon liver malic enzyme was prepared according to Hsu & Lardy (1967), specific activity, 28 units/mg. protein; lactate dehydrogenase activity [assayed according to Neilands (1955)], 0.05 μmoles lactate oxidized per min. per mg. protein.

System	mμmoles $NADPH_2$ or $NADH_2$ oxidized/min.		Mg^{2+} (or Mn^{2+}) dependent $NADPH_2$ (or $NADH_2$) oxidation mμmoles/min.
	without Mg^{2+} (1)	with Mg^{2+} (or Mn^{2+}, bottom line) (2)	(2) - (1)
Complete	1.4	54.0	52.6
Complete, with 75 μg. enzyme	1.0	26.0	25.0
$NADPH_2$ omitted, $NADH_2$, 0.64 μmole, added	6.8	6.0	0
Pyruvate omitted	0.5	0.5	0
$MgCl_2$ omitted, $MnCl_2$, 18 μmoles, added*	1.0	68.5	67.5

* Crystalline bovine albumin was not added.

The product of pyruvate reduction was identified as L-lactate by the specific enzymic assay for L-lactate (Hohorst, 1963) as shown in Table 2. The formation of near stoicheiometric amounts of L-lactate suggests that reaction 3 accounts for the metal-activated $NADPH_2$ oxidation. In other experiments the small discrepancy between $NADPH_2$ oxidized and lactate formed was accounted for by the formation of malate, presumably by reversal of reaction 1 due to small amounts of CO_2 in the reaction mixture. Thus far, attempts to demonstrate the reduction of NADP by L-lactate at pH 7 or 10 have not been successful. The possibility that the slow turnover of reaction 3 is catalysed by a separate enzyme contaminating the crystalline malic enzyme preparation cannot be unequivocally ruled out by the present results. However, the requirement for both $NADPH_2$ and a divalent metal is compelling circumstantial evidence in favour of the pyruvate reductase activity residing in malic enzyme. Furthermore, possession of activity for reaction 3 is consistent with the

mechanism of malic enzyme predicted by product inhibition studies[1] on reaction 1, where bicarbonate is released first, and a malic enzyme-pyruvate-$NADPH_2$ complex is formed. This ternary complex is common to reactions 1 and 3, and could break down to form either pyruvate and $NADPH_2$ as in reaction 1, or, more slowly, to L-lactate and NADP as in reaction 3 depending on the conditions of the experiment.

Table 2

Stoicheiometry of L-*lactate formation*

Reaction mixtures for $NADPH_2$ oxidation are the same as described in Table 1. Temperature, 25°. After 20 min., the reaction was stopped by the addition of 1.0 ml. 10% trichloroacetic acid. Protein precipitate was removed by centrifugation for 10 min. The supernatant solution was neutralized with 0.06 ml. of 5 M-K_2CO_3, and an aliquot (0.5 ml.) was assayed for L-lactate by the method of Hohorst (1963), using crystalline lactate dehydrogenase from muscle. L-Lactate values are corrected by a reagent blank in the initial incubation for $NADPH_2$ oxidation. This reagent blank contained all reagents, but malic enzyme was added after acidification with trichloroacetic acid.

All spectrophotometric measurements were made at 340 mμ in a Beckman Model DU Spectrophotometer.

Experiment	System	$NADPH_2$ oxidized (μmoles)	L-Lactate formed (μmoles)
1a	Complete	0.321	0.305
1b	$MgCl_2$ omitted	0.031	0.065
	a — b	0.290	0.240
2a*	Complete	0.376	0.361
2b*	$MgCl_2$ omitted	0.031	0.041
	a — b	0.345	0.320

* 0.96 μmoles $NADPH_2$ was used.

Our present results also clarify the early findings of Kaufman, Korkes & Del Campillo (1951) that free pyruvate is not an intermediate in the formation of L-lactate from L-malate *via* reaction 4 catalysed by a preparation of NAD-specific malic enzyme from *Lactobacillus arabinosus*. Kaufman *et al.* assumed that a lactate dehydrogenase-malic enzyme complex accomplished the over-all reaction without participation of free pyruvate. Their results might be explained better by the assumption that the malic enzyme of *L. arabinosus* is capable of carrying out reaction 4 without the intervention of lactate dehydrogenase.

$$\text{L-Malate} \overset{\text{NAD, } Mn^{2+}}{\rightleftharpoons} \text{L-lactate} + CO_2 \qquad (4)$$

The physiological significance of reaction 3 catalysed by the crystalline malic enzyme is not clear. Because of its low rate, reaction 3 may not be expected to play

[1] Hsu, R.Y. & Lardy, H.A., manuscript to be submitted.

an important part in the cellular functions of pigeon liver, unless under *in vivo* conditions this activity is elevated significantly.

This work was supported by grants from the National Science Foundation and the National Institutes of Health.

REFERENCES

Hohorst, H.J. (1963). In *Methods of Enzymatic Analysis* (H.U. Bergmeyer, ed.) p. 266. Academic Press Inc., New York.

Hsu, R.Y. & Lardy, H.A. (1967). *J. Biol. Chem.* **242**, 520.

Kaufman, S., Korkes, S. & Del Campillo, A. (1951). *J. Biol. Chem.* **192**, 301.

Neilands, J.B. (1955). In *Methods in Enzymology* (S.P. Colowick & N.O. Kaplan, eds.) Vol. I, p. 449. Academic Press Inc., New York.

„ENZYM JABŁCZANOWY" Z WĄTROBY GOŁĘBIA

IV. AKTYWNOŚĆ REDUKTAZY PIROGRONIANOWEJ KRYSTALICZNEGO ENZYMU

Streszczenie

1. Stwierdzono, że krystaliczna dehydrogenaza jabłczanowa (dekarboksylująca) z wątroby gołębia katalizuje $NADPH_2$ specyficzną redukcję pirogronianu na L-mleczan, przy czym powstaje NADP. Reakcja aktywowana jest przez Mg^{2+} lub Mn^{2+}.

2. Wykazano stechiometrię tej reakcji.

3. Szybkość redukcji pirogronianu wynosi około 1% aktywności tlenowej dekarboksylacji jabłczanu.

Received 27 September, 1966.

ACTA BIOCHIMICA POLONICA
Vol. XIV 1967 No. 1

RECENZJE KSIĄŻEK

Dittrich F.L.: BIOPHYSICS OF THE EAR. American Lectures Series. C.C. Thomas, Publ., Springfield, Ill., U.S.A. 1963; str. 189.

Jest faktem bezspornym, że od pewnego czasu o dalszym postępie medycyny decydują nie lekarze, a specjaliści innych dziedzin nauki. Dittrich (podobnie jak Bekesy) jest fizykiem i próbuje ująć fizjologię ucha od strony zjawisk fizycznych przy pomocy reguł matematycznych.

Jest to pierwsza publikacja książkowa ujmująca całość zagadnienia fizyki ucha w sposób ścisły. Książka dzieli się na cztery części. W pierwszej i drugiej autor przedstawia historię rozwoju wiedzy o anatomii i fizjologii ucha, mechanikę ślimaka, zagadnienie percepcji dźwięków oraz czułość narządu słuchu. W części trzeciej, poświęconej błędnikowi, autor zajmuje się mechaniką przedsionkową, sprawą równowagi oraz pobudzenia i przesyłania bodźców w układzie utrzymania równowagi. W tej zwłaszcza części użyto dużą ilość wzorów i formuł matematycznych, co utrudnia zrozumienie nieprzygotowanemu matematycznie czytelnikowi.

Część czwarta omawia elementy cybernetyki ślimaka i przedsionka. Autor przedstawia elektroniczne modele układu słuchowego i równowagi, dochodząc w końcu do wniosku, że cudowność urządzeń żywych jest nieporównywalna.

Całość stanowi pracę bardzo ciekawą, chociaż trudną; pozwala ona spojrzeć na zagadnienia otologiczne pod zupełnie innym kątem widzenia niż ten, do którego przywykliśmy w codziennej pracy, i prowadzi do lepszego zrozumienia zagadnień fizjologii ucha.

J. Iwaszkiewicz

MOLECULAR BIOLOGY OF MUSCULAR CONTRACTION (S. Ebashi, F. Oosawa, T. Sekine and Y. Tonomura, eds.) Elsevier Publ. Co., Amsterdam - London - New York 1965; str. 206+ XII, cena DFl. 55,—

Książka, wydana pod redakcją czołowych japońskich biochemików zajmujących się biochemią mięśnia szkieletowego, stanowi dziewiąty tom biblioteki *Biochimica et Biophysica Acta*. Jest to zbiór prac dedykowanych profesorowi Hiroshi Kumagai z okazji jego wycofania się z czynnej pracy na Uniwersytecie Tokijskim. Zamieszczone prace pochodzą z wielu pracowni japońskich i amerykańskich, a napisane są przez badaczy, którzy zetknęli się z profesorem Kumagai.

Od kilkunastu lat obserwujemy, że Japonia staje się jednym z krajów przodujących w badaniach nad biochemią mięśnia; jak się dowiadujemy ze wstępu do omawianej książki, H. Kumagai, jakkolwiek sam zajmował się farmakologią centralnego układu nerwowego, to jednak uważany jest przez młodych badaczy japońskich za twórcę podstaw sukcesów japońskich w dziedzinie badań nad mięśniami. Pod kierunkiem H. Kumagai i w jego pracowni wykryto w roku 1955 uorganizowany czynnik rozkurczowy mięśni.

Prace zamieszczone w książce podzielono na sześć grup, którym nadano następujące tytuły: teoretyczne podejście do funkcji mięśnia; struktura i funkcja miozyny A; chemia fizyczna aktyny; interakcja ATP - aktomiozyna; porównawcze aspekty funkcji mięśnia; oraz sprzężenie pobudzenia i skurczu poprzez retikulum sarkoplazmatyczne. Niektóre artykuły zawierają ciekawe teoretyczne uogólnienia, w innych (jak np. w ładnie napisanej pracy W.W. Kielley'a p.t. *Studies on the Structure of Myosin*) znajdujemy propozycje rozwiązań także już wcześniej publikowane przez ich autorów.

Wiele z zamieszczonych w książce prac zawiera nowe interesujące dane z zakresu różnorodnych zagadnień biochemii mięśnia. Zebranie ich w jednym tomie jest nie tylko uczczeniem zasłużonego uczonego, ale także cenną inicjatywą dla tych wszystkich, którzy chcieliby zapoznać się z ważniejszymi kierunkami współczesnych badań nad biochemią mięśnia.

Książka powinna zainteresować nie tylko badaczy bezpośrednio zaangażowanych w doświadczenia nad mięśniami, ale także szerszy ogół biochemików, fizjologów i farmakologów.

Mariusz Żydowo

Lissak K. and Endröczi E.: THE NEUROENDOCRINE CONTROL OF ADAPTATION. Pergamon Press, Oxford 1965; str. 180, cena 70 $.

Omawiana książka jest kolejnym 25-ym tomem serii monografii poświęconych czystej i stosowanej biologii. Dotyczy ona wyników badań nad współzależnością pomiędzy ośrodkowym układem nerwowym a układem gruczołów dokrewnych. Książka składa się z dziewięciu rozdziałów, przy czym każdy zaopatrzony jest w wykaz literatury często przekraczający sto pozycji.

Pierwszy rozdział traktuje bardzo krótko o anatomicznych połączeniach pomiędzy układem nerwowym centralnym a przysadką mózgową z uwzględnieniem funkcjonalnych połączeń z korą nadnerczy. W drugim rozdziale omówiono podstawy metabolizmu hormonów kory nadnerczy oraz regulację ich wydzielania. Biosyntezę kortykosterydów ujęto w cztery etapy: zamianę C_{27} sterydu w C_{21} steryd, wytworzenie grupy Δ^4-3-ketonowej w pierścieniu A, powstanie łańcucha bocznego dwuhydroksyacetonu i hydroksylacji w pozycji C_{17} i C_{11} pierścienia sterydowego. Należałoby może dla ścisłości podać, że etap hydroksylacji w pozycji C_{11} następuje po etapie hydroksylacji w pozycji C_{21}. W podanym przez autorów schemacie biosyntezy hormonów sterydowych omyłkowo przedstawiono możliwość zamiany przy udziale ACTH kortyzolu w cholesterol (błędnie narysowany wzór). Metabolizm hormonów sterydowych — coprawda marginesowy dla zagadnień poruszanych w tej książce — oparto na danych doświadczalnych sprzed 10 lat. Regulację wydzielania kortykosterydów rozpatrzono głównie dla aldosteronu (mylnie podanego jako 11,21-dwuhydroksy-3,20-dwuketo-4-pregnen-18-ol) i roli regulacyjnej ACTH dla wydzielania kortykosterydów. W trzecim rozdziale, dotyczącym specyficznego przystosowania funkcji układu przysadkowo-nadnerczowego, przedstawiono głównie wyniki własnych doświadczeń. Omówiono stosowane metody oznaczania kortykosterydów. Jako błąd drukarski należy przyjąć określenie czułości metod oznaczania i identyfikacji wynoszące 0.5 g. dla 17,21-dwuhydroksykortykosterydów i 0.25 g. na cm^2 dla Δ^4-3-ketosterydów. Autorzy piszą, że w doświadczeniach *in vitro* użyto płyn inkubacyjny z dodatkiem 0.1 μM-ATP i 0.25 μM-fumaranu sodu, który zwiększał aktywność enzymatyczną, gdy substratem był progesteron, kortyzon i kortyzol. Jaką aktywność enzymatyczną mają autorzy na myśli, tego nie można wywnioskować z dalszych wyników. W dwóch układach chromatograficznych dokonują autorzy rozdziału i identyfikacji 19 kortykosterydów kory nadnerczy. Nasuwa się wątpliwość, jak dalece ścisłe mogą być kryteria użyte do identyfikacji tych związków. Wyniki własne przedstawione w tym rozdziale obejmują wpływ kortykosterydów, hormonów płciowych, tyroksyny na wydzielanie kortykosterydów nadnerczowych. Czwarty rozdział dotyczy hormonalnej regulacji uwalniania ACTH. Część tego rozdziału poświęcona jest metodyce oznaczania ACTH, natomiast pozostałą część stanowią rozważania na temat wpływu adrenaliny i histaminy na uwalnianie ACTH. W piątym rozdziale przedstawiono dane dotyczące związków czynnościowych pomiędzy podwzgórzem a przysadką, ze szczególnym uwzględnieniem wydzielania ACTH. Szósty rozdział traktuje o funkcji układu nadnerczowo-przysadkowego w czasie rozwoju. Cytowane fakty doświadczalne nasuwają przypuszczenie o braku zależności pomiędzy ACTH matki a funkcją hormonalną nadnerczy u płodu. Natomiast poziom kortykosterydów we krwi matki warunkuje wydzielanie ACTH płodowego, a tym samym reguluje czynność układu przysadkowo-nadnerczowego u płodu. Rozdziały VII i VIII dotyczą kontroli układu przysadkowo-nadnerczowego przez centralny układ nerwowy. Zawierają one również spostrzeżenia własne autorów oraz wiele danych z literatury. Wyniki doświadczeń cytowane w tych rozdziałach są często sprzeczne. Jest to zrozu-

miałe, jeżeli uwzględni się metodykę ogólnie stosowaną w tego rodzaju badaniach. Technika elektrokoagulacji, pobudzanie elektryczne, lokalne stosowanie hormonów oraz usuwanie pewnych części anatomicznych mózgu — to zabiegi, które mogą dać wieloznaczne wyniki. W ostatnim rozdziale omówiono działanie hormonów na centralny układ nerwowy. Jest szereg danych wskazujących na zmiany w układzie nerwowym centralnym pod wpływem kortykosterydów. Nie wiadomo dotąd, w jaki sposób działają na układ nerwowy estrogeny i progesteron. Godne uwagi są spostrzeżenia własne autorów dotyczące istnienia związku pomiędzy odruchami warunkowymi a poziomem kortykosterydów we krwi zwierząt doświadczalnych.

W omawianej książce autorzy próbowali dokonać przeglądu zagadnień związanych z neuroendokrynologiczną regulacją w oparciu o piśmiennictwo do roku 1960 i wyniki badań własnych. Nagromadzone w tej książce fakty doświadczalne, często sprzeczne ze sobą, nie pozwalają jeszcze teraz na wyrobienie sobie jednolitego poglądu na udział ośrodkowego układu nerwowego w regulacji hormonalnej.

Leon Żelewski

Heilmeyer L. (przy współpracy R. Clottena i L. Heilmeyera, Jr.) DISTURBANCES IN HEME SYNTHESIS (tłumaczenie z niemieckiego M. Steinera) C.C. Thomas Publ., Springfield, III., U.S.A. 1966; str. 236, cena 10.50 $.

Książka wydana jest w serii American Lecture Series i omawia mechanizm biosyntezy hemu w warunkach fizjologicznych oraz zaburzenia tej syntezy w różnych typach anemii i innych schorzeniach. Monografia obejmuje 7 rozdziałów, w których autorowie omawiają kolejno m.in. kliniczny obraz zaburzeń syntezy hemu, główne przyczyny tych zaburzeń, przebieg syntezy protohemu w warunkach prawidłowych, metody analityczne dla wykrywania zaburzeń syntezy hemu u pacjentów oraz szczegółowe objawy patologiczne przy niewłaściwym przebiegu biosyntezy porfiryn.

Przebieg biosyntezy hemu w warunkach prawidłowych jest omówiony szczegółowo począwszy od cyklu glicynowo-bursztynowego i tworzenia kwasu δ-aminolewulinowego, poprzez jego kondensację do porfobilinogenu, następnie proces kondensacji prowadzący do powstania połączeń 2-, 3-, 4-pyrolowych, wytworzenie pierścienia oktapyrolowego, powstawanie uroporfirynogenu, który w końcu poprzez szereg stadiów zamieniany jest w protoporfirynę IX. Również dość wyczerpująco omówione są poszczególne reakcje enzymatyczne prowadzące do powstania protohemu, lokalizacja enzymów oraz aktywatory i inhibitory kolejnych etapów biosyntezy.

W rozdziale V omówione są metody analizy chemiczno-klinicznej: oznaczanie żelaza, badania za pomocą radioaktywnego izotopu żelaza ^{59}Fe, oznaczanie wolnych porfiryn oraz ich prekursorów w erytrocytach krwi obwodowej, w moczu i szpiku kości. Następnie omówiono oznaczanie kwasu δ-aminolewulinowego, porfobilinogenu, jak również opisano szereg doświadczeń nad biosyntezą porfiryn, w tym oznaczanie aktywności niektórych enzymów biorących udział w procesie biosyntezy. Podano również opisy rozdziału porfiryn za pomocą chromatografii i wysokonapięciowej elektroforezy bibułowej.

Następny rozdział poświęcony jest patologii syntezy hemu. Opisano liczne doświadczenia przeprowadzane zarówno *in vivo*, jak i *in vitro* w przypadkach różnych odmian anemii, z omówieniem mechanizmu tych zaburzeń, objawów klinicznych i zmian morfologicznych. Podane są również możliwości leczenia niektórych schorzeń w oparciu o poznany mechanizm zaburzenia. Autorowie klasyfikują przyczyny zaburzeń biosyntezy hemoglobiny w zależności od: braku żelaza w ustroju, braku protoporfiryny IX oraz braku odpowiedniej ilości globiny. Ten ostatni przypadek prawdopodobnie ma miejsce w thalassemii. Proces łączenia się trzech podstawowych składników barwnika krwi zachodzi w mitochondriach jądrzastych erytrocytów pod wpływem swoistej syntetazy hemu (włączenie żelaza do porfiryny) oraz innych enzymów. Autorowie opisują liczne przykłady zaburzeń tego procesu, choć ich badania własne dotyczyły głównie przemian pomiędzy kwasem δ-aminolewulinowym a protoporfiryną. W badaniach tych uwzględniano również problemy genetyczne.

Książka napisana jest przejrzyście, cały materiał jest doskonale usystematyzowany, przez co czyta się ją lekko, choć traktuje niejednokrotnie o zawiłych problemach biochemicznych i klinicz-

nych. Porusza wiele zagadnień czysto biochemicznych, jak i będących na pograniczu teorii i praktyki klinicznej, przez co jest interesująca zarówno dla teoretyka, jak i klinicysty, a zwłaszcza hematologa.

Włodzimierz Ostrowski

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS, vol. VIII, part 3: Chromatography (ed. T. Holman), Pergamon Press, Oxford 1966; str. 119, cena 6.50 $.

Kolejny tom Progress... przynosi trzy artykuły poświęcone metodyce chromatografii lipidów. W pierwszym z nich D.C. Malins omawia ostatnie osiągnięcia w dziedzinie chromatografii cienkowarstwowej, zajmując się szczególnie możliwościami rozdziału lipidów o bardzo zbliżonej budowie. W dwu tabelach autor zestawia kilkadziesiąt procedur rozdziałów chromatograficznych na płytkach adsorbenta impregnowanych rozpuszczalnikami hydrofobowymi oraz rozdziałów na płytkach pokrywanych mieszaninami adsorbentu z różnymi czynnikami kompleksującymi (azotan srebra, kwas borowy, arsenin sodu). Przy użyciu tych dwu technik stało się możliwe rozdzielenie wszystkich „krytycznych par" związków lipidowych. Omówione są tu również różne metody ilościowego oznaczania substancji rozdzielonych metodą chromatografii cienkowarstwowej.

W krótkim artykule poświęconym chromatografii bibułowej lipidów J.G. Hamilton omawia jej zastosowanie do rozdziału kwasów tłuszczowych, tłuszczów obojętnych i fosfolipidów. W odniesieniu do lipidów chromatografia bibułowa została w dużej mierze wyparta przez chromatografię cienkowarstwową i mimo iż obecnie wiele firm oferuje różnego typu gotowe impregnowane bibuły chromatograficzne, przy pomocy których można uzyskać rozdziały nie gorsze niż na płytkach, ujemną stroną tej procedury pozostaje stosunkowo długi czas rozwijania chromatogramów oraz niemożność zastosowania testu zwęglania do lokalizacji plam.

W ostatnim artykule p.t. *Chromatografia kolumnowa lipidów* (R.A. Stein & Vida Slawson) omówiono różne typy stosowanych adsorbentów i sposoby ich przygotowywania. Bardzo pożyteczne dla praktyki laboratoryjnej są obszerne zestawienia charakterystyki pojemności różnego typu kolumn chromatograficznych dla różnych klas lipidów oraz ok. 300 przykładów rozdziału różnych lipidów na kolumnach różnych adsorbentów.

Wśród sporej ilości innych monografii poświęconych szybko rozwijającym się technikom chromatografii lipidów omawiana pozycja zajmuje wysoką rangę i stanie się użytecznym nabytkiem we wszystkich laboratoriach lipidowych.

Tadeusz Chojnacki

Jaques L.B.: ANTICOAGULANT THERAPY. PHARMACOLOGICAL PRINCIPLES. C.C. Thomas Publ., Springfield, Ill. U.S.A. 1965; str. 156, cena 7.50 $.

Dr L.B. Jaques, kierownik Katedry Fizjologii i Farmakologii Uniwersytetu w Saskatchewan, Saskatoon, Kanada, zajmuje się zagadnieniami farmakologii antykoagulantów już ponad 30 lat i jest jednym z pionierów w tej dziedzinie. Omawiana książka jest podsumowaniem wyników wieloletnich badań jego zespołu. Jest pisana jasno, przystępnie, posiada duże walory dydaktyczne. Obejmuje pięć rozdziałów: 1) farmakologia i antykoagulanty; 2) antykoagulanty i choroba zakrzepowa — autor omawia tu pokrótce patogenezę choroby zakrzepowej i podstawy farmakologiczne stosowania antykoagulantów; 3) heparyna — szczegółowo omówione są tu własności chemiczne, metody izolowania i oznaczanie heparyny, a także jej rozmieszczenie w tkankach i komórkach, jej różnorodne działanie biologiczne oraz zasady leczniczego stosowania heparyny; 4) antykoagulanty pośrednie, t.j. antykoagulanty z grupy dikumarolu i phenylindanedionu; omówiono tu pokrótce mechanizm działania tych leków i zasady ich stosowania oraz dość szczegółowo metabolizm dikumarolu w ustroju; 5) samoistne krwawienie pod wpływem antykoagulantów. Rozdział ten poświęcony jest omówieniu własnych badań autora na ten temat. Zdaniem Jaquesa zarówno heparyna, jak i dikumarol nie wywołują *per se* u zwierząt doświadczalnych żadnych krwawień, o ile nie dołączy się jakiś dodatkowy czynnik upośledzający hemostazę, np. stress, usu-

nięcie nadnerczy, uszkodzenie krwinek płytkowych. Na podstawie tych badań autor przedstawia własną koncepcję hemostazy.

Książka L.B. Jaquesa przedstawia zagadnienie terapii antykoagulantami i ich farmakologii w sposób niewątpliwie dość jednostronny, nie wzmiankowano nawet wielu bardzo istotnych poglądów i prac z tego zakresu (np. praca O'Reilly i Aggellera na temat uwarunkowanej genetycznie oporności na pochodne dikumarolu u ludzi, prace Ratnoffa dotyczące blokowania heparyną różnych czynników krzepnięcia, mechanizm leczniczego działania heparyny w emboliach płucnych, etc.). Książka L.B. Jaquesa daje odnośniki jedynie do 43 pozycji piśmiennictwa, co ogranicza jej użyteczność w pracach naukowych.

Stefan Niewiarowski

CYCLITOLS AND PHOSPHOINOSITIDES. Proceedings of the 2nd Meeting of the Federation of European Biochemical Societies, Vienna, 21 - 24 April 1965, Vol. 2 (H. Kindl, ed.). Pergamon Press, Oxford 1966; str. 80, cena 20 s.

Omawiany tom jest zbiorem referatów przedstawionych na kollokwium poświęconym cyklitolom i fosfoinozytydom, zorganizowanym w ramach II-go Kongresu F.E.B.S. w Wiedniu w 1965 r. Kollokwium to było pierwszą tego rodzaju konferencją poświęconą temu zagadnieniu, a zawarte w książce materiały stanowią dobrą i wyczerpującą monografię zagadnienia. Wśród autorów opublikowanych referatów widnieją nazwiska wszystkich bardziej znanych badaczy zajmujących się cyklitolami i fosfoinozytydami.

W rozdziale I *Biosynteza inozytolu u ssaków* F. Eisenberg, Jr. (National Institutes of Health, Bethesda, U.S.A.) omawia mechanizm biosyntezy inozytolu z glukozy, w którym glukozo-6-fosforan ulega cyklizacji przy współudziale NAD z wytworzeniem, pod wpływem enzymu z jąder szczura, inozytolo-1-fosforanu, który następnie jest defosforylowany.

W rozdziale *Biosynteza mezo-inozytolu u mikroorganizmów i roślin wyższych* H. Kindl (Organisch-Chemisches Institut der Universität, Vienna, Austria) przedstawia badania nad mechanizmem tej biosyntezy przy użyciu D-glukozy znakowanej ^{14}C i ^{3}H w różnych położeniach. W rozdziale tym szeroko potraktowano metody chemicznej odbudowy inozytolu. Uzyskane wyniki świadczą, iż mechanizm cyklizacji glukozy jest prawdopodobnie jedyną drogą powstawania inozytolu w naturze.

O. Hoffmann-Ostenhof (Organisch-Chemisches Institut der Universität, Vienna, Austria) omawia biosyntezę cyklitoli innych niż mezo-inozytol. Szczególnie dużą rozmaitość tych związków znaleźć można u roślin. Zagadnienie rodzaju prekursora tych cyklitoli nie zostało dotychczas wyjaśnione.

Th. Posternak (Laboratoires de Chimie biologique et organique speciale de l'Université, Geneve, Szwajcaria) przedstawił badania nad analogami strukturalnymi inozytolu będącymi jego antagonistami u *Neurospora crassa* i *Schizosaccharomyces pombe*. W pracowni autora przebadano ponad 60 tego rodzaju połączeń. W referacie omówione są efekty morfonogenetyczne wywołane przez antagonistów inozytolu oraz przypuszczalny mechanizm biochemiczny ich działania.

C. Ballou i Y.C. Lee (Department of Biochemistry, University of California, U.S.A.) omawiają budowę chemiczną glicerylofosforyloinozytolomannozydów występujących u mykobakterii.

W końcowych trzech rozdziałach, których autorami są: J. N. Hawthorne i R. H. Michell (University of Birmingham, Anglia), R.M.C. Dawson (Institute of Animal Physiology, Cambridge, Anglia) i R.J. Rossiter i F.B. Palmer (University of Western Ontario, London, Kanada) przedstawiono i omówiono najnowsze wyniki badań nad biosyntezą i funkcją fosfoinozytydów w układzie nerwowym i w innych tkankach.

Książka zaopatrzona jest w indeks rzeczowy. Stanowić ona może bardzo interesującą i pomocną lekturę dla biochemików zajmujących się cyklitolami i fosfolipidami.

Tadeusz Chojnacki