POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

Vol. 21 No. 2

QUARTERLY

ABPLAF 21 (2) 113–234 (1974)

WARSZAWA 1974

PWN POLISH SCIENTIFIC PUBLISHERS

POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

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QUARTERLY

PWN POLISH SCIENTIFIC PUBLISHERS
WARSZAWA 1974

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Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakład 1049+111 egz. Ark. wyd. 10,25 ark. druk. 8,0+0,375.
Papier druk. sat. kl. III, 80 g. 70×100
Oddano do składania 8.III.74 r. Podpisano do druku 13.VII.74 r.
Druk ukończono w lipcu 1974
Zam. 196 W-121 Cena zł 25.—

Warszawska Drukarnia Naukowa, Warszawa, Śniadeckich 8

ACTA BIOCHIMICA POLONICA
Vol. 21 1974 No. 2

ALEKSANDRA KUBICZ, BRONISŁAWA MORAWIECKA and M. KRUZEL

HETEROGENEITY OF ACID PHOSPHATASE FROM POTATO TUBERS

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Heterogeneity of the non-specific acid phosphatase (EC 3.1.3.2) from potato tubers has been demonstrated by disc electrophoresis, isoelectric focusing and adsorption on calcium phosphate gel. On disc electrophoresis, the acid phosphatase separated into three activity zones, irrespective of the preparation method used and degree of enzyme purification. The multiple forms of the enzyme showed different isoelectric points in the pH range 5.4 - 6.6, and different affinity to calcium phosphate gel.

By DEAE-cellulose chromatography Felenbok (1970) demonstrated heterogeneity of acid phosphatase from bean sprouts, and Verjée (1969) of the enzyme present in wheat germs. Kubicz & Morawiecka (1970, 1971) showed by polyacrylamide-gel electrophoresis the heterogeneity of acid phosphatase from potato tubers, and Kubicz (1973) reported that the observed multiple molecular forms of the enzyme had the same molecular weight but differed in charge. In the present work, the multiple forms of potato acid phosphatase were shown to be independent of the mode of preparation and degree of purification of the enzyme.

EXPERIMENTAL

Material

For preparation of acid phosphatase, white potatoes of the variety "Alma" were used. Ampholine (pH 5 - 8) was from LKB-Produkter AB (Bromma 1, Sweden); other reagents were from the same sources as in the previous work (Kubicz, 1973), and acrylamide and bisacrylamide were recrystallized from chloroform as described by Loening (1967).

Methods

Enzyme preparations. Preparation I was obtained after Mejbaum-Katzenellenbogen & Morawiecka (1959); the potato tubers were homogenized with 0.9% NaCl

and the extract concentrated by the tannin-caffeine method (Mejbaum-Katzenellenbogen, 1955). The obtained protein solution was dialysed, freeze-dried and subjected to Sephadex G-75 chromatography (Kubicz, 1973); the preparation was tenfold purified, and its specific activity, tested with sodium phenylphosphate as substrate, was about 2.4 units/mg. The yield was 21%.

Preparation II was obtained by the method of Hsu *et al.* (1966). The protein was extracted with acetate buffer of pH 3.4 and twice fractionated with tannin. From the tannin-protein sediment, the tannin was removed with acetone. The specific activity of preparation II tested with *p*-nitrophenylphosphate as substrate was 30 units/mg, the purification being about 100-fold and the yield about 0.2%.

Adsorption on calcium phosphate gel. The gel was prepared according to Keilin & Hartree (Colowick, 1955). To 1.5 ml of the gel (45 mg dry wt.), 10 mg of protein (preparation I) dissolved in 1 ml of 0.1 M-acetate buffer, pH 5.0, was applied; after 20 min at 4°C, the mixture was centrifuged, and the fluid (F_1) was collected. The sediment was washed with 1 ml of the above buffer, centrifuged, the adsorbed protein extracted with 1 ml of 0.01 M-sodium citrate, pH 7.0, and the fluid obtained after centrifugation called F_2. The solutions F_1 and F_2 were dialysed against water.

Polyacrylamide-gel electrophoresis was carried out at 4°C for 1.5 - 2 h, at pH 9.5 according to Ornstein (1964) and Davis (1964), or at pH 4.3 according to Reisfeld *et al.* (1962), as described previously (Kubicz, 1973).

Isoelectric gel focusing. This was performed according to Righetti & Drysdale (1971) in 4% and 6% polyacrylamide gels containing 2% ampholine. The gel tubes were cooled by circulating water, and the whole apparatus was immersed in ice-water. Following electrophoresis, from the gels to be used for zymograms the ampholine was removed by diffusion to 0.2 M-acetate buffer (twice for 20 min), then the enzymic activity was located. For pH gradient determination, the gels were cut into 5 mm sections which were dispersed in 1.5 ml water, left overnight at 4°C in tightly closed tubes, then pH was determined using a microelectrode of the pH-meter (Radiometer, Kopenhagen, Denmark).

Acid phosphatase activity determination was made as described previously (Kubicz 1973) using as substrate phenylphosphate (for preparation I) or *p*-nitrophenylphosphate (for preparation II). One unit of the enzyme corresponds to 1 μmol of decomposed substrate per 1 mg. In the gels, the activity of the enzyme was detected using α-naphthol phosphate and Fast Blue B as described previously (Kubicz, 1973).

Protein in the solutions was estimated by the tannin method (Mejbaum-Katzenellenbogen, 1955), and in the gels it was located by staining with Amido Black.

RESULTS AND DISCUSSION

Irrespective of the mode of preparation and the degree of purification, the two preparations of acid phosphatase both at acid and alkaline pH values gave on disc electrophoresis three zones of activity (Fig. 1). Each of the active zones separated into 2 - 3 subfractions which were especially pronounced on the electrophoretograms

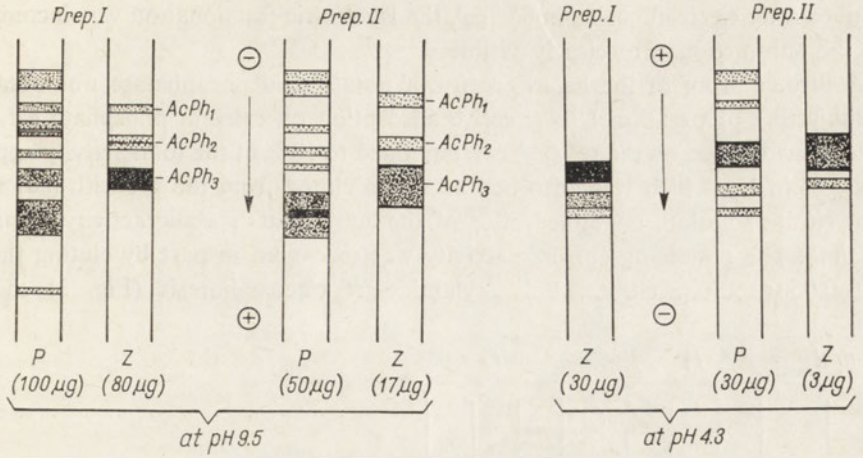

Fig. 1. Polyacrylamide-gel electrophoresis of acid phosphatase preparations I and II at acid and alkaline pH values. *P*, proteinogram; *Z*, zymogram; the amount of protein applied to the gel is indicated in parentheses. For details see Methods.

from alkaline electrophoresis shown in Fig. 3 (control zymogram). Both in preparation I and II, fraction $AcPh_3$, corresponding to acid phosphatase III, was the dominant form.

Electrofocusing of preparation I showed that the observed differences in charge between particular molecular forms of potato acid phosphatase (Kubicz, 1973) were reflected in differences in their isoelectric points. In Fig. 2 are presented the zymograms obtained after electrofocusing in 4% and 6% gels, and the dependence of mobility on the pH gradient. The isoelectric points of the molecular forms of the enzyme lie within the pH range 5.4 - 6.6. In this range, the particular components were clearly separated showing different isoelectric points. In the zymogram

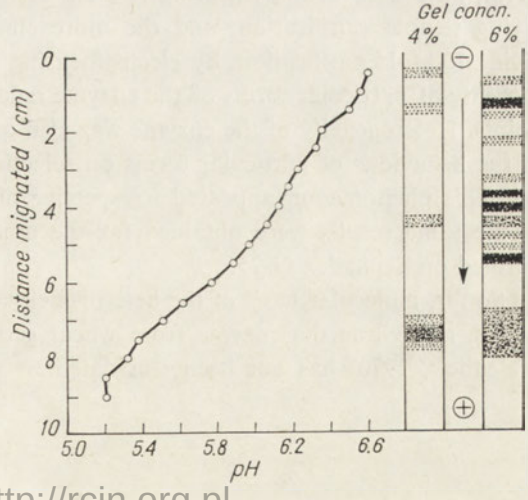

Fig. 2. Isoelectric fractionation of potato acid phosphatase. Preparation I, 250 µg of protein, was subjected to electrofocusing on 4% and 6% polyacrylamide gels, and enzyme activity was located; in the 4% gel, pH was also determined. For details see Methods.

obtained after electrofocusing in 6% gel, the isoelectric fractionation was incomplete but the subfractions are clearly visible.

A further proof of the heterogeneity of potato acid phosphatase was obtained by subjecting preparation I to selective adsorption on calcium phosphate gel. The protein unadsorbed on the gel (F_1) corresponded to 10% of the total activity applied. The protein F_2, which was adsorbed and then eluted from the gel with 0.01 M-sodium citrate solution, contained 50% of the activity, its specific activity being 5.5 units/mg. The remaining enzymic activity was recovered in part by eluting the gel with 0.05 M-sodium citrate. Polyacrylamide-gel electrophoresis (Fig. 3) showed

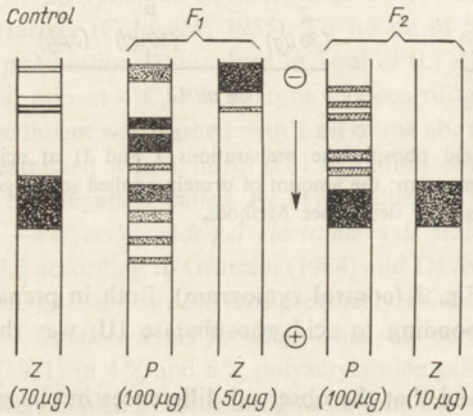

Fig. 3. Electrophoresis on 7.5% polyacrylamide gel at pH 9.5 of acid phosphatase preparation I unadsorbed (F_1) and adsorbed (F_2) on calcium phosphate gel. *P*, proteinogram; *Z*, zymogram; the amount of protein applied to the gel is indicated in parentheses. For comparison, the zymogram of preparation I not subjected to calcium phosphate gel adsorption (control) is given.

that, of the activity zones present in the starting preparation I, F_1 contained the slower-migrating forms, whereas the dominating, fast-migrating form of the acid phosphatase was found in F_2.

The previous results showing the occurrence of multiple forms of acid phosphatase from potato tubers differing in charge but not in molecular weight (Kubicz, 1973), have been confirmed using two extraction methods: that applied previously, giving tenfold purification, and the more efficient method of Hsu *et al.* (1966), giving 100-fold purification. By electrofocusing it was found that differences in the mobility of particular forms of the enzyme reflect the differences in their isoelectric points. Heterogeneity of the enzyme was also confirmed by the observed differences in the behaviour of particular forms on selective adsorption on calcium phosphate gel. This phenomenon appeared irrespective of the degree of enzyme purification, as the same results were obtained for the crude preparation and the preparation purified on Sephadex G-75.

So far, molecular basis of the heterogeneity of the acid phosphatase from potato tubers, as well as the enzyme from wheat germs (Verjée, 1969) and bean sprouts (Felenbok, 1970) has not been elucidated.

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HETEROGENNOŚĆ KWAŚNEJ FOSFATAZY ZIEMNIAKA

Streszczenie

Heterogenność niespecyficznej kwaśnej fosfomonoesterazy (EC 3.1.3.2) ziemniaka wykazano za pomocą elektroforezy dyskowej, izoelektrycznego frakcjonowania oraz adsorpcji na żelu fosforanowo-wapniowym. Preparaty kwaśnej fosfatazy, niezależnie od metody preparacji i stopnia oczyszczenia, dzielą się w elektroforezie na trzy strefy o aktywności enzymatycznej. Poszczególne formy molekularne enzymu różnią się punktem izoelektrycznym w zakresie pH of 5.4 do 6.6, jak również zdolnością do adsorpcji na żelu fosforanowo-wapniowym.

Received 21 September, 1973.

ACTA BIOCHIMICA POLONICA

Vol. 21 1974 No. 2

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THE ROLE OF MALATE IN THE CLEAVAGE OF CHOLESTEROL SIDE-CHAIN IN THE COURSE OF PROGESTERONE BIOSYNTHESIS IN HUMAN TERM PLACENTA

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1. Biosynthesis of [^{14}C]progesterone from [^{14}C]cholesterol in the human term placenta mitochondrial fraction was found to be malate- and $NADP^+$-dependent, and was enhanced by Mn^{2+}. Malate could not be replaced by its metabolites, pyruvate or oxaloacetate. Pregnenolone, an intermediate of progesterone biosynthesis, was not detectable. 2. Formation of [^{14}C]progesterone from [^{14}C]pregnenolone in the mitochondrial fraction was not affected by malate or $NADP^+$.

Malate plays an important role as a factor controlling steroidogenesis in ovary (Robinson & Stevenson, 1971) and adrenals (Simpson & Estabrook, 1969; Sauer & Park, 1973). This is due to the mitochondrial malic enzyme activity which results in the generation of NADPH, a cofactor essential for the cytochrome P-450 dependent mixed function oxidases. Mitochondria from human placenta possess a second electron-transport chain concerned with steroid-hydroxylation reactions and with cholesterol side-chain cleavage (Ryan *et al.*, 1966; Mason & Boyd, 1971). This electron-transport chain, like that associated with steroid hydroxylation in adrenal cortex and ovary mitochondria is using NADPH as the reducing source.

The occurrence of the malic enzyme activity in human term placenta mitochondrial fraction (Aleksandrowicz *et al.*, 1971) suggested that, like in adrenals and ovary, malate plays an important role in the system controlling placental steroidogenesis. Bogusławski *et al.* (1972) demonstrated that malate indeed stimulated biosynthesis of progesterone from cholesterol. The present work is concerned mainly with another step of progesterone synthesis: the conversion of pregnenolone (3β-hydroxypregn-5en-20-one) to progesterone (pregn-4-ene-3,20-dione) in human term placenta mitochondrial fraction.

MATERIALS AND METHODS

Reagents. The chemicals used were obtained from the following sources: $NADP^+$ from Boehringer Corp. (London, England); L-malic acid and oxaloacetic acid from

Koch-Light Lab. (Colnbrook, Bucks., England); D-glucose-6-phosphate (G-6-P), G-6-P dehydrogenase (200 - 400 units/mg protein) and pyruvic acid from Sigma Chem. Co. (St. Louis, Mo., U.S.A.); [4-^{14}C]cholesterol (spec. act. 58 mCi/mmol), [4-^{14}C]pregnenolone (spec. act. 51 mCi/mmol), [^{3}H]pregnenolone (spec. act. 6.9 Ci/mmol) and [^{3}H]progesterone (spec. act. 255 mCi/mmol) from the Radiochemical Centre (Amersham, England). All other chemicals used were analytical grade products of Polish origin.

Preparation of mitochondrial fraction from human term placenta was performed as described in the previous work (Bogusławski *et al.*, 1972).

Assay for the conversion of cholesterol to pregnenolone and progesterone. The incubations were carried out for 1 h at 37°C in air with constant shaking. The incubation mixture contained in the final volume of 2.5 ml: 20 mM-potassium phosphate buffer, pH 7.4, 10 mM-magnesium sulphate, 1 mM-manganese sulphate, 1 mM-$NADP^{+}$ and 0.5 ml mitochondrial suspension in 0.154 M-KCl (20 mg protein). The reaction was initiated by the addition of ^{14}C-labelled substrate, cholesterol or pregnenolone, and terminated by the addition of 10 ml of methanol containing a known amount of [^{3}H]pregnenolone and [^{3}H]progesterone, or [^{3}H]progesterone alone. The tritiated compounds were added to check the recovery of progesterone or pregnenolone, which was calculated on the basis of tritium found in the final product.

Extraction and isolation of ^{14}C-labelled steroids. The protein precipitate was centrifuged down and reextracted with boiling ethyl acetate. Chloroform was added to the combined extracts and the mixture was centrifuged to separate the organic and aqueous phases. The organic phase was taken to dryness, progesterone and pregnenolone were added as "cold" carriers, and chromatographed on thin layer plates of silica gel impregnated with rhodamine 6-G in system *I*: methylene chloride-ethyl ether (5:2, v/v). The pregnenolone and progesterone bands were eluted and further purified in systems: *II*, benzene - ethanol (9:1, v/v); *III*, benzene - ethyl acetate (3 : 2, v/v) and *IV*, methylene chloride - methanol (98:2, v/v). Final crystallization of progesterone and pregnenolone was carried out in *n*-hexane - acetone mixture (25:1, v/v).

In some experiments, the dry residue of the organic phase was saponified with 15% KOH in methanol at 60°C for 2 h. The solution was neutralized with acetic acid using phenolphthalein. The saponified extract was evaporated and the residue partitioned between chloroform and water. The chloroform fraction was evaporated and subjected to t.l.c. as described above.

Radioactivity assay. The radioactivity was counted in 10 ml of scintillation fluid containing 4 g of 2,5-diphenyloxazole and 0.2 g of 1,4-di[2(5-phenyloxazolyl)]benzene per litre of toluene, using Nuclear Chicago Mark I spectrometer.

Analytical. Protein was determined by the biuret method (Layne, 1957) and cholesterol colorimetrically with the Liebermann-Burchard colour reagent (Sperry & Webb, 1950).

RESULTS AND DISCUSSION

The time-course of [^{14}C]progesterone biosynthesis from [^{14}C]cholesterol by the human term placenta mitochondrial fraction, in the presence of optimum concentrations of malate and $NADP^+$, and the enhancing effect of Mn^{2+} is shown in Fig. 1. Throughout the whole incubation period only radioactive progesterone was formed, whereas no [^{14}C]pregnenolone, which is regarded as an intermediate

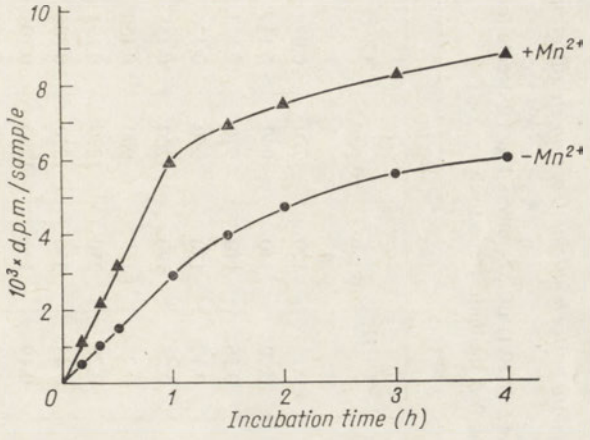

Fig. 1. Time course of progesterone formation from cholesterol in the human placental mitochondrial fraction, and the effect of Mn^{2+}. The incubation was carried out with 20 mg of mitochondrial protein in 2.5 ml of medium (pH 7.4) containing 0.15 μCi of [4-^{14}C]cholesterol, 20 mM-potassium phosphate, 10 mM-$MgSO_4$, 1 mM-$NADP^+$, 10 mM-malate and, where indicated, 1 mM-$MnSO_4$.

in progesterone synthesis, could be detected in the incubation medium (Table 1). The supposition that pregnenolone could have accumulated in the form of esters was excluded by the analysis of the saponified incubation medium. It is possible that the placental mitochondrial fraction used in our studies was contaminated with microsomes to a higher degree, or that 3β-hydroxysteroid dehydrogenase and Δ^{4-5}isomerase activity was higher in our preparation than in the mitochondrial fraction from adrenals or ovary used by Hall (1972) and by Robinson & Stevenson (1971), respectively. It cannot be excluded, either, that placental mitochondria, like those of rat testis (Sulimovici *et al.*, 1973) contain these two enzymes.

The conversion of [^{14}C]pregnenolone to [^{14}C]progesterone in the placental mitochondrial fraction (Table 2) was lower than that obtained by Ryan *et al.* (1966) but higher than the conversion found by Sobrevilla *et al.* (1964) in homogenates of placental tissue. This high conversion of pregnenolone to progesterone is consistent with the lack of pregnenolone accumulation during the biosynthesis of progesterone from cholesterol. In contrast to progesterone formation from cholesterol, malate and $NADP^+$ had no effect on the conversion of pregnenolone.

The formation of progesterone from pregnenolone exceeded many times the conversion of [^{14}C] cholesterol in the presence of malate with $NADP^+$, or NADP-regenerating system (Table 3). This suggests that the former reaction is not the rate-

Table 1

Assay for pregnenolone formation from [^{14}C]cholesterol in the human placenta mitochondrial fraction at different times of incubation

The incubation mixture was as described in Methods, the content of [4-^{14}C]cholesterol being 0.15 μCi. Numbers 1 and 2 denote the pregnenolone fractions extracted from the non-saponified and saponified medium, respectively.

Time of incubation		I t.l.c.			II t.l.c.			III t.l.c.			IV t.l.c.	
		^{14}C (d.p.m.)	^{3}H (d.p.m.)	^{14}C/^{3}H	^{14}C (d.p.m.)	^{3}H (d.p.m.)	^{14}C/^{3}H	^{14}C (d.p.m.)	^{3}H (d.p.m.)	^{14}C/^{3}H	^{14}C (d.p.m.)	^{3}H (d.p.m.)
5 min	1	602	500	1.20	140	900	0.16	120	1220	0.098	*	815
	2	343	390	0.88	120	580	0.20	140	1040	0.134	*	850
10 min	1	860	600	1.43	160	980	0.16	110	970	0.113	*	780
	2	630	450	1.40	120	700	0.17	130	950	0.136	*	690
20 min	1	1020	605	1.68	140	560	0.25	190	1200	0.158	*	750
	2	280	540	0.52	120	520	0.23	135	900	0.150	*	660
30 min	1	980	640	1.53	260	940	0.28	250	1290	0.193	*	765
	2	1550	510	3.04	400	940	0.43	350	1220	0.286	*	620
60 min	1	1050	650	1.61	160	840	0.19	135	1280	0.105	*	550
	2	430	600	0.72	400	990	0.40	290	1140	0.254	*	660

* The counts of ^{14}C did not exceed twice the background values.

Table 2

Conversion of [^{14}C]pregnenolone to [^{14}C]progesterone in the human placenta mitochondrial fraction

The incubation mixture was as described in Methods except that NADP$^+$ was omitted; each sample contained 0.08 μCi of [4-^{14}C]pregnenolone.

Additions	IV t.l.c.			Crystals			[^{14}C]Progesterone (d.p.m./sample)	Conversion (%)
	^{14}C (d.p.m.)	^{3}H (d.p.m.)	^{14}C/^{3}H	^{14}C (d.p.m.)	^{3}H (d.p.m.)	^{14}C/^{3}H		
None, control	21 000	3250	6.46	16 000	2700	5.94	77 200	44
NADP$^+$, 1 mM	28 540	4470	6.38	20 900	3350	6.24	81 100	46
NADP$^+$, 1 mM + malate, 10 mM	15 180	2560	5.93	12 800	2240	5.70	74 100	42
Malate, 10 mM	18 640	3000	6.21	21 150	3550	5.95	77 300	44

Table 3

The effect of malate, pyruvate and oxaloacetate on progesterone formation from [^{14}C]cholesterol in the human placenta mitochondrial fraction

The incubation mixture was as described in Methods except that NADP$^+$ was omitted; the content of [^{14}C]cholesterol was 0.15 μCi.

Additions	Progesterone biosynthesis							
	I t.l.c.	II t.l.c.	III t.l.c.	Crystals				
	^{14}C/^{3}H	^{14}C/^{3}H	^{14}C/^{3}H	^{14}C (d.p.m.)	^{3}H (d.p.m.)	^{14}C/^{3}H	^{14}C (d.p.m./sample)	conversion (%)
None, control	0.50	0.10	0.08	*	290		0	0
Malate, 10 mM	0.78	0.15	0.11	*	360		0	0
Malate, 10 mM + NADP$^+$, 1 mM	2.27	1.73	1.68	810	500	1.62	6 100	1.8
Pyruvate, 10 mM + NADP$^+$, 1 mM	0.47	0.12	0.10	*	520		0	0
Oxaloacetate, 10 mM + NADP$^+$, 1 mM	0.54	0.12	0.10	*	560		0	0
G-6-P, 10 mM + G-6-P dehydrogenase, 1 unit + NADP$^+$, 1 mM	6.20	5.35	5.08	1850	370	5.00	19 000	5.7

* The counts of ^{14}C did not exceed twice the background values.

-limiting step in progesterone biosynthesis. The highest value of [^{14}C]cholesterol conversion to progesterone observed in the human placental mitochondrial fraction by Mason & Boyd (1971) and in our experiments, was about 6%. The mitochondrial fraction contained about 28 mg of total endogenous cholesterol per gram of mitochondrial protein. The non-esterified cholesterol fraction, accounting for 24 mg/g of mitochondrial protein, diluted the added radioactive cholesterol, and resulted in a lower value of the found incorporated radioactivity, whereas in fact a large part of the non-radioactive cholesterol was converted to progesterone.

Formation of progesterone from cholesterol was much lower in the presence of 10 mM-malate and $NADP^+$ (Table 3) than in the presence of a system regenerating reducing equivalents. This suggests that the capacity of placental mitochondria to reduce $NADP^+$ in the presence of optimum malate concentration was lower than the requirement of reducing equivalents for the maximum conversion of cholesterol to progesterone. No progesterone biosynthesis was observed when malate without $NADP^+$ was added, or the possible metabolites of malate, like pyruvate and oxaloacetate, were added together with $NADP^+$.

The presented evidence supports the assumption that in placental mitochondria the malic enzyme system may play an important role in the cleavage of cholesterol side-chain.

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UDZIAŁ JABŁCZANU W REAKCJI ROZSZCZEPIENIA ŁAŃCUCHA BOCZNEGO CHOLESTEROLU W PROCESIE BIOSYNTEZY PROGESTERONU W DOJRZAŁYM ŁOŻYSKU LUDZKIM

Streszczenie

1. Zwiększona biosynteza [^{14}C]progesteronu z [^{14}C]cholesterolu zależna od jabłczanu i $NADP^+$ jest stymulowana przez Mn^{2+} we frakcji mitochondrialnej łożyska ludzkiego. Metabolity jabłczanu: pirogronian i szczawiooctan są bez wpływu na badany etap steroidogenezy. Nie wykazano radioaktywności w pregnenolonie — metabolicie biosyntezy progesteronu z cholesterolu.

2. Biosynteza [^{14}C]progesteronu z [^{14}C]pregnenolonu we frakcji mitochondrialnej łożyska jest niewrażliwa na obecność jabłczanu oraz $NADP^+$.

Received 3 October, 1973.

ACTA BIOCHIMICA POLONICA
Vol. 21 1974 No. 2

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PURIFICATION AND PROPERTIES OF AMP-AMINOHYDROLASE FROM HUMAN PLACENTA *

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1. A method resulting in 570-fold purification of AMP-aminohydrolase from human placenta is presented. 2. The purified enzyme was inactivated by dialysis against 1 mM-EDTA; reactivation has been observed upon addition of some divalent cations: Zn^{2+}, Mn^{2+} and Co^{2+}. 3. The enzyme was activated by Li^{+} ions and ATP, whereas inorganic phosphate exerted a strong inhibitory effect. 4. The kinetic and regulatory properties of human placental AMP-aminohydrolase resemble those of the enzyme from avian and calf brain, and differ from those of the enzyme from skeletal muscle.

AMP-aminohydrolase (EC 3.5.4.6) is a very widespread enzyme, the highest activity of which was observed in skeletal muscle. The enzyme from this source has been obtained in crystalline form (Smiley *et al.*, 1967) and its catalytic and physico-chemical properties thoroughly investigated (Zielke, 1970; Zielke & Suelter, 1971a). Apart from skeletal muscle, partially purified preparations of AMP-aminohydrolase have so far been obtained from avian and calf brain (Henry & Chilson, 1969; Setlow & Lowenstein, 1967), rat liver (Smith & Kizer, 1969) and avian erythrocytes (Henry & Chilson, 1973). Comparative studies have also been performed with the enzyme from erythrocytes of rabbit (Razin & Mager, 1966), man (Askari & Franklin, 1965; Askari, 1966; Rao *et al.*, 1968) and chicken (Henry & Chilson, 1973). Chicken erythrocyte showed 15 times higher activity as compared with rabbit and human erythrocytes investigated under similar experimental conditions (Henry & Chilson, 1973).

It has been demonstrated previously (Makarewicz & Maćkowiak, 1971) that human placenta contains specific 5′-AMP-aminohydrolase (EC 3.5.4.6). In this paper, partial purification and some properties of the purified enzyme are reported.

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

METHODS

Determination of AMP-aminohydrolase activity. The enzyme activity was determined from the amount of ammonia liberated from 5′-AMP assayed either directly or following microdiffusion which was necessary when the imidazole buffer, which interferes with ammonia determination, was used. The phenol-hypochlorite reaction performed according to Chaney & Marbach (1962) was used for determination of ammonia. The incubation mixture contained in a final volume of 2 ml: 0.1 M-imidazole-HCl or 50 mM-K-succinate buffer, pH 6.5, 5′-AMP (imidazole or K salt) and other compounds at concentrations indicated in the legends to Tables and Figures. The reaction was started by the addition of 25 or 50 µl of the enzymic preparation. Incubation was carried out in test tubes at 30°C. After appropriate time intervals (10, 20 and 30 min, or 20, 40 and 60 min), the reaction was stopped either by adding 1 ml of 15% (w/v) trichloroacetic acid (TCA) followed after 10 min by neutralization with 1 ml of 0.9 M-KOH, or by transferring 0.5 ml sample to the microdiffusion bottle containing 1 ml of saturated potassium carbonate solution. In the first case, ammonia was assayed after centrifugation directly in 1 ml samples of the clear supernatant. In the second case, microdiffusion of ammonia was performed at 37°C overnight according to Seligson & Seligson (1951) with the modification introduced by Strelkov (1967). The microdiffusion bottles contained 1 ml of saturated potassium carbonate solution, and after introduction of the samples were immediately closed with rubber stoppers in which a glass rod with an end moistened in 1 N-H_2SO_4 solution had been placed. Ammonia collected in minute amounts of 1 N-H_2SO_4 on the end of the glass rod in the microdiffusion bottle was rinsed to a test tube with 5 ml of phenol reagent. Hypochlorite reagent (5 ml) was then added and the colour developed at 37°C for 30 min. The extinction was read at 620 nm with a Coleman-14 spectrophotometer. Two parallel incubations were carried out and the mean rate of the reaction was calculated.

Due to the low activity of placental AMP-aminohydrolase in the absence of ATP under our experimental conditions, the spectrophotometric assay at 265 nm could not be employed.

Protein determination. This was made either by the method of Lowry *et al.* (1951) using as standard fraction V of bovine serum albumin, or spectrophotometrically as described by Layne (1957).

Reagents, 5′-AMP was supplied by E. Merck AG (Darmstadt, West Germany), and ATP by Austranal (Wien, Austria). Imidazole was from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., England), Tris from Fluka AG (Buchs, Switzerland), bovine serum albumin, fraction V from Sigma Chem. Co. (St. Louis, Mo., U.S.A.), caesium chloride, lithium chloride, 2-mercaptoethanol and Amberlite IR-120 were from British Drug Houses (Poole, Dorset, England). Cellulose phosphate, P-1, capacity 7.4 mequiv./g was from Whatman Biochemicals Ltd. (Maidstone, Kent, England), and Sephadex G-25 and G-200 from Pharmacia Fine Chemicals (Uppsala,

Sweden). Other reagents were supplied by Polskie Odczynniki Chemiczne (Gliwice, Poland).

Glass-redistilled water was used throughout. In the analytical procedure, water was additionally freed of ammonia on Amberlite IR-120.

RESULTS

Purification of AMP-aminohydrolase

The course of enzyme purification is outlined in Table 1. All preparative steps were carried out at 4°C. Stages 1 and 2 were performed according to Smiley *et al.* (1967) with minor modifications.

Stage 1: preparation of tissue extract. Fresh human placentae freed of membranes were scraped with scissors and the tissue (1000 - 2000 g) was washed three times with 5 vol. of 0.9% NaCl, blotted on filter paper, disintegrated in a meat grinder and homogenized for 2 min at full speed in a Waring Blendor with 2 vol. of 0.089 M-K-phosphate buffer, pH 6.5, containing 0.18 M-KCl and 1 mM-2-mercaptoethanol (extraction buffer). The homogenate was stirred for 1 h and then centrifuged for 1 h at 1300 *g*.

Stage 2: absorption on phosphocellulose. To the obtained supernatant, phosphocellulose pulp (5 - 10 g dry wt.) soaked in the extraction buffer was added and left overnight with gentle stirring. Then cellulose phosphate containing the absorbed

Table 1

Purification of AMP-aminohydrolase from human placenta

The enzyme activity in the extract was estimated after overnight dialysis against 400 vol. of 1.0 M-KCl containing 1 mM-2-mercaptoethanol. In the assay of AMP-aminohydrolase, the incubation mixture contained in a final volume of 2 ml: 50 mM-K-succinate buffer, pH 6.5, 100 mM-KCl and 10 mM-5'-AMP (potassium salt). After incubation for 10, 20 and 30 min the reaction was stopped by the addition of 1 ml of 15% TCA, the samples were treated as described under Methods, and ammonia was measured directly without microdiffusion.

Protein in the extract was measured colorimetrically (Lowry *et al.*, 1951) and at further stages of purification, spectrophotometrically at 280 and 260 nm (Layne, 1957). The ratio E_{260}/E_{280} of the eluate from phosphocellulose was usually about 1.0.

Stage	Total protein (mg)	Total activity (μmol/min)	Spec. act. (μmol/min/mg of protein)	Yield (%)	Purification
1. Extract	46 600	2520	0.054	100	—
2. Eluate from phosphocellulose column	96	397	4.13	16	76
3. Ppt. at 0 - 1.85 M-ammonium sulphate	26	133	5.11	5.3	85
4. Sephadex G-200 gel filtration	1.7	52	30.6	2.1	570

enzyme was allowed to settle. The obtained slurry was washed on a Buchner funnel with approximately 2 litres of the extraction buffer and then with 1 litre of 0.45 M-KCl solution adjusted to pH 7.0 with 1 M-K_2HPO_4 containing 1 mM-2-mercaptoethanol. After washing, the slurry was transferred to a column (2 × 40 cm), washed further with 200 ml of 0.45 M-KCl, pH 7.0, and the enzyme was eluted with 1.0 M-KCl, pH 7.0, containing 1 mM-2-mercaptoethanol.

Stage 3: fractionation with ammonium sulphate. To the combined active fractions eluted from the column, solid ammonium sulphate was added slowly up to 1.85 M concentration at 4°C with constant stirring. After 3 h the precipitate containing AMP--aminohydrolase was collected by centrifugation, dissolved in a small volume of 1.0 M-KCl, pH 7.0, containing 1 mM-2-mercaptoethanol and dialysed overnight against the same solution to remove excess of ammonium ions.

Stage 4: gel filtration on Sephadex G-200. After dialysis the small amount of insoluble protein was centrifuged off and the clear supernatant was applied to a Sephadex G-200 column equilibrated with 1.0 M-KCl, pH 7.0, containing 1 mM-2--mercaptoethanol. One peak of activity appearing after the main peak of protein migrating at the void volume (V_0) was obtained on eluting the column with the same solution (Fig. 1).

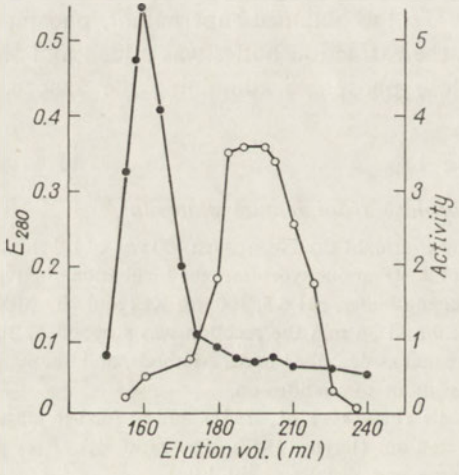

Fig. 1. Sephadex G-200 gel filtration of placental AMP-aminohydrolase preparation (step 4, Table 1). The column (2.5 × 90 cm) was equilibrated and eluted at 4°C with 1 M-KCl containing 1 mM-2-mercaptoethanol. The flow rate was 12 ml/h, 3 ml fractions were collected. ○, Enzymic activity expressed in μmol NH_3/min/3 ml of eluate (for experimental conditions see legend to Table 1). ●, E_{280}.

The purified enzyme did not contain 5′-nucleotidase (EC 3.1.3.5) or adenosine aminohydrolase (EC 3.5.4.4), and was stable for 2 - 3 weeks when stored at 4°C. For the experiments with activators, the enzyme solution was desalted using a column (0.8 × 30 cm) packed with Sephadex G-25 coarse, and transferred into 0.3 M-succinate-Tris buffer, pH 6.5.

Properties of AMP-aminohydrolase

Prolonged dialysis of the purified enzyme against 1.0 M-KCl buffered with 50 mM-imidazole-HCl, pH 6.5, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol, resulted in a complete loss of activity. Similarly as with the muscle enzyme

(Zielke, 1970; Zielke & Suelter, 1971b; Raggi *et al.*, 1971), the activity could be restored by adding some divalent cations to the incubation medium. Full reactivation of the enzyme was observed in the presence of Zn^{2+} at a concentration of 50 μM, but some activity was also observed on addition of Co^{2+}, Mn^{2+}, Cu^{2+} and Cd^{2+} (Table 2). The addition of Fe^{2+}, Mg^{2+} or Ca^{2+} was ineffective. When the enzyme preparation was dialysed against the solution without EDTA, the addition of Zn^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+} or Mn^{2+} had no effect on the enzyme activity.

The effect of pH on the rate of 5′-AMP deamination catalysed by the purified placental enzyme is shown in Fig. 2. The highest reaction rate was observed at pH 6.5.

The purified enzyme responded also to the addition of monovalent cations (Table 3). At the substrate concentration of 5 mM, in the absence of monovalent

Table 2

Effect of divalent cations on the activity of EDTA-inactivated placental AMP-aminohydrolase

The enzyme solution was dialysed for 24 h at 4°C against 250 volumes of 50 mM-imidazole-HCl buffer, pH 6.5, containing 1000 mM-KCl, 1 mM-2-mercaptoethanol and 1 mM-EDTA. In control dialysis EDTA was omitted. The enzyme activity was assayed in 100 mM-imidazole-HCl buffer, pH 6.5, containing 1 mM-ATP, 5 mM-5′-AMP (imidazole salt) and divalent cations at indicated concentrations. Fe^{2+} and Co^{2+} were added as sulphates, other cations as chlorides. Ammonia liberated during incubation for 20, 40 and 60 min was assayed after microdiffusion. Figures represent the relative rates of reaction assuming as 100 the rate of the reaction catalysed by the enzyme dialysed without EDTA and assayed without any divalent cation added. Data are mean values from the number of experiments indicated in parentheses.

Concentration (μM)	Zn^{2+}	Co^{2+}	Cd^{2+}	Cu^{2+}	Mn^{2+}	Fe^{2+}	Mg^{2+}	Ca^{2+}
10	13 (4)	26 (3)	22 (3)	12 (2)	0 (2)	0 (2)	0 (2)	0 (2)
25	65 (1)	50 (1)	—	—	54 (1)	—	—	—
50	100 (4)	55 (4)	11 (3)	20 (2)	43 (2)	0 (2)	0 (2)	0 (2)
100	93 (1)	50 (1)	—	—	54 (1)	—	—	—

Table 3

Effect of monovalent cations on the activity of placental AMP-aminohydrolase

The activity was estimated in 100 mM-imidazole-HCl buffer, pH 6.5. Monovalent cations were added in the form of chlorides at concentration of 150 mM, and 5′-AMP (imidazole salt) at concn. of 5 mM. The reaction was started by the additon of 25 μl of desalted enzyme preparation purified up to stage 3 (cf. Table 1). The rate of reaction in the presence of Li^+ was taken as 100. Data are mean values from the number of experiments indicated in parentheses ±S.D.

Cation added	Activity
None	4.8±2.1 (3)
Li^+	100.0 (3)
Na^+	60.0±8.7 (3)
K^+	46.0±5.1 (3)
Cs^+	6.0 (2)

cations the enzyme exhibited very low activity, whereas in the presence of Li^+ the increase in activity was the greatest (about 20-fold). The optimum concentration of Li^+ in the incubation mixture was found to be 150 mM (Fig. 3). On addition of Na^+ or K^+ the activity was approximately half that with Li^+, whereas Cs^+ had practically no activating effect.

AMP-aminohydrolase was also activated by ATP (Fig. 4), the maximum activation being observed at a concentration as low as 1 mM.

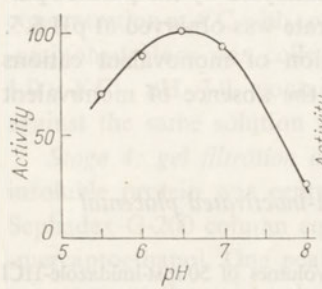

Fig. 2

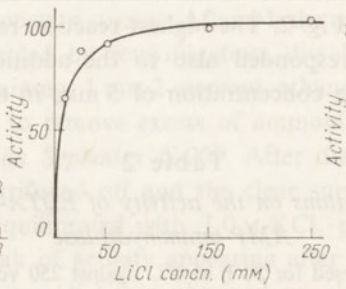

Fig. 3

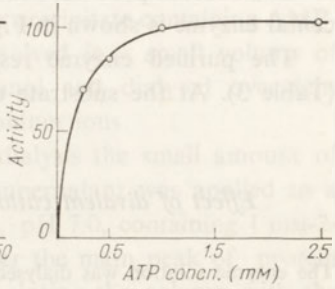

Fig. 4

Fig. 2. Optimum pH for the activity of placental AMP-aminohydrolase. The incubation mixture contained in a final volume of 1 ml: 100 mM-imidazole-HCl buffer, 150 mM-KCl and 10 mM-5′-AMP (imidazole salt). Ammonia liberated after incubation for 10, 20 and 30 min was measured. Each point represents the mean from two experiments performed in triplicate. Relative activity was calculated assuming the rate of reaction at pH 6.5 as 100.

Fig. 3. Effect of Li^+ on the activity of placental AMP-aminohydrolase. Incubation was carried out in 100 mM-imidazole-HCl buffer, pH 6.5, for 10, 20 and 30 min. 5′-AMP (imidazole salt) concentration was 5 mM. The reaction was started by the addition of 25 µl of desalted enzyme preparation. Each point represents the mean from 2 experiments performed in duplicate each. Relative activity was calculated assuming the rate of reaction in the presence of 150 mM-LiCl as 100.

Fig. 4. Effect of ATP on the activity of placental AMP-aminohydrolase. Relative activity calculated assuming the reaction rate in the presence of 1 mM-ATP (imidazole salt) as 100. Each point represents the mean from 2 - 3 experiments. For experimental conditions see Fig. 3.

The effect of substrate concentration on the rate of reaction in the absence (Fig. 5) and in the presence of Li^+ and ATP (Fig. 6) was investigated using the incubation mixture consisting of 50 mM-K-succinate buffer, pH 6.5, 100 mM-KCl and indicated concentrations of 5′-AMP (potassium salt); or 100 mM-imidazole-HCl buffer, pH 6.5, 5′-AMP (imidazole salt) at concentrations indicated, and 1 mM-ATP (imidazole salt) or 150 mM-LiCl.

In the presence of K^+ ions, the enzyme gives a sigmoidal *v versus* S plot (Fig. 5A) and a non-linear $1/v$ *versus* $1/S$ plot (not shown). The interaction coefficient n calculated from the slope of Hill plot (Dawes, 1972) equals 2.5 (Fig. 5B). The activating effect of both Li^+ and ATP is compared in Fig. 6 and Table 4. In these experiments K^+ ions were absent from the incubation mixture and therefore the activity of the control mixture was very low. It is apparent from Fig. 6A that at any substrate concentration studied, the enzyme activity in the presence of ATP was higher than in the presence of Li^+, the difference being greater at lower substrate concentrations.

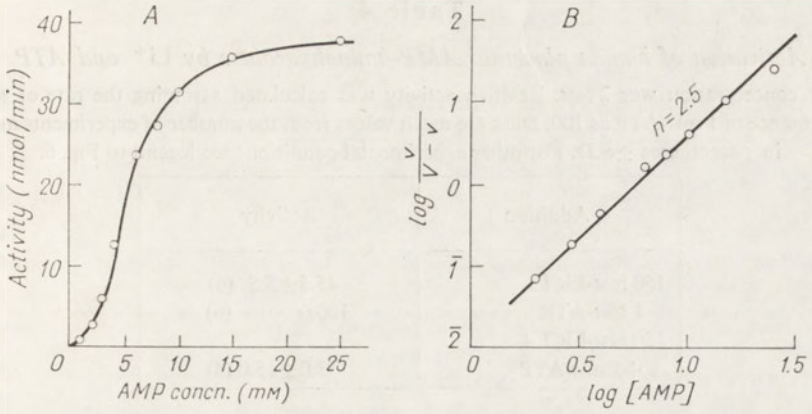

Fig. 5. Effect of substrate concentration on the rate of the reaction catalysed by human placental AMP-aminohydrolase. *A*, *v versus* S plot; *B*, log $v/(V-v)$ *versus* log S plot (Hill plot). Maximal velocity for calculation of log $v/(V-v)$ was assumed as 39 nmol/min from plot (*A*). Enzyme activity was assayed as described in the legend to Table 1.

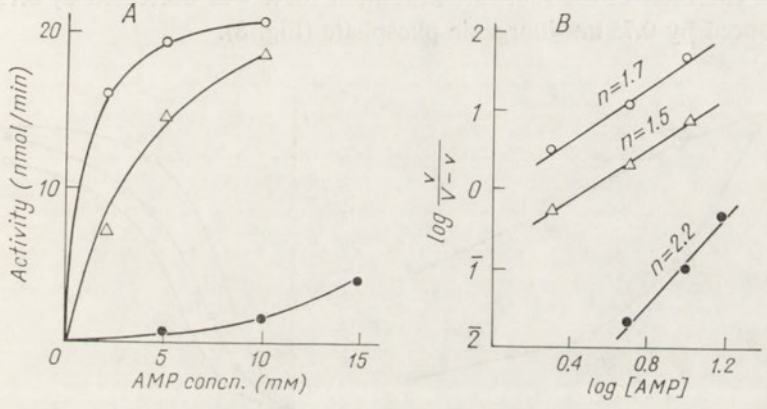

Fig. 6. Kinetics of Li^+ and ATP activation of AMP-aminohydrolase. *A*, *v versus* S plot in the absence of activators (●), and in the presence of 1 mM-ATP (○), or 150 mM-LiCl (△). *B*. Hill plot of log $v/(V-v)$ *versus* log S. For calculation of log $v/(V-v)$ maximum velocity was assumed as 21.2 nmol/min/10 μg of protein. The incubation mixture contained: 100 mM-imidazole-HCl buffer, pH 6.5, 5′AMP (imidazole salt) at concentrations indicated and 1 mM-ATP (imidazole salt), or 150 mM-LiCl. Final volume 2 ml. The reaction was started by the addition of 25 - 100 μl of desalted enzyme preparation purified up to stage 3 (Table 1). The mean rate of reaction was calculated from three different periods of incubation performed in duplicate.

When both Li^+ and ATP were present in the incubation medium, the rate of the reaction was but slightly higher than in the presence of ATP alone (Table 4). The interaction coefficient *n* (Fig. 6B) was 2.2 in the absence of ATP and Li^+, and 1.7 and 1.5 in the presence of ATP and Li^+, respectively. The value of *n* was lower in the presence of Li^+ than in the presence of K^+ which indicates that in the presence of Li^+ the enzyme had greater affinity towards the substrate.

Table 4

Activation of human placental AMP-aminohydrolase by Li^+ *and ATP*

5′-AMP concentration was 2 mM. Relative activity was calculated assuming the rate of reaction in the presence of 1 mM-ATP as 100. Data are mean values from the number of experiments indicated in parentheses ±S.D. For other experimental conditions see legend to Fig. 6.

Addition	Activity
150 mM-LiCl	45.5±5.5 (6)
1 mM-ATP	100.0 (6)
150 mM-LiCl + +1 mM-ATP	133.0±15.0 (4)

Placental AMP-aminohydrolase is strongly inhibited by inorganic phosphate (Fig. 7). The extent of inhibition depends on the presence of ATP and Li^+. Inorganic phosphate in concentration of 1 mM inhibited in 25% the activity of the enzyme in the presence of ATP, and in 80% in the presence of Li^+. In the presence of 0.1 M-KCl, sigmoidal character of the substrate saturation curve was decreased by 0.15 M-LiCl, and enhanced by 0.75 mM-inorganic phosphate (Fig. 8).

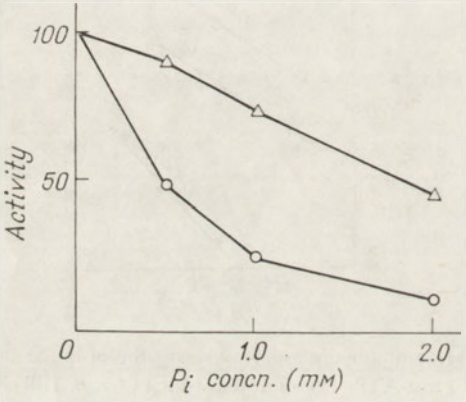

Fig. 7

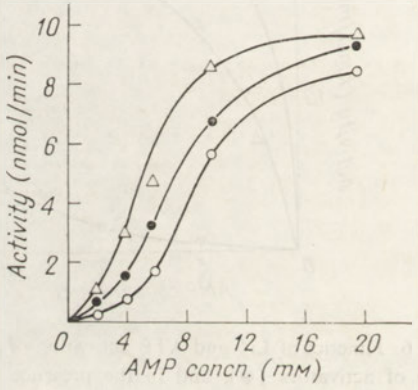

Fig. 8

Fig. 7. Inhibition of placental AMP-aminohydrolase by inorganic phosphate. The incubation mixture contained in a final volume of 2 ml: 100 mM-imidazole-HCl buffer, pH 6.5, 2 mM-5′-AMP (imidazole salt), 1 mM-ATP (△) or 150 mM-LiCl (○) and inorganic phosphate in concentrations indicated. The reaction was started by the addition of 50 µl of enzyme solution in 0.3 M-Tris-succinate buffer, pH 6.5. Each point represents the mean from two experiments performed in duplicate. Relative activity was calculated assuming the rate of reaction in the absence of inorganic phosphate as 100.

Fig. 8. Kinetics of the inhibition of placental AMP-aminohydrolase by inorganic phosphate. The incubation mixture contained in a final volume of 1 ml: 150 mM-imidazole-HCl buffer, pH 6.5, 100 mM-KCl, 5′-AMP (imidazole salt) at concentration indicated, and 150 mM-LiCl (△) or 0.75 mM-inorganic phosphate (imidazole salt) (○). Control (●) without LiCl or inorganic phosphate added. The reaction was starte d by the addition of 0.1 ml of enzyme purified up to stage 3 (Table 1). Each point represents the mean reaction rate calculated from three time intervals.

DISCUSSION

The placental AMP-aminohydrolase has been purified about 570-fold. The activity of the preparation obtained (about 30 μmol/min/mg protein) is of the same order of magnitude as that obtained from avian brain (Henry & Chilson, 1969) and about twice as high as that from calf brain (Setlow & Lowenstein, 1967). The activity of crystalline AMP-aminohydrolase from rabbit skeletal muscle (Zielke & Suelter, 1971a) is 1200 - 1600 μmol/min/mg protein.

Comparison of the properties of AMP-aminohydrolase isolated from various sources shows differences in substrate affinity and response to cations and ATP. In the absence of activators the rate of reaction catalysed by placental AMP-aminohydrolase at lower substrate concentrations was very low (Fig. 6) and therefore the enzyme activity could not be assayed spectrophotometrically unless ATP was added. Very similar behaviour has been reported for chicken brain enzyme (Henry & Chilson, 1973). The preparation of placental enzyme resembles that of brain also with respect to activation by monovalent cations (Table 3). Both enzymes show the highest activity in the presence of Li^+ (Setlow & Lowenstein, 1967; 1968; Henry & Chilson, 1973). Na^+ and K^+ activate the enzyme half as effectively (Setlow & Lowenstein, 1967). ATP activates placental AMP-aminohydrolase to a greater extent than monovalent cations (Fig. 6, Table 4); the same is true for brain enzyme (Setlow & Lowenstein, 1967; Henry & Chilson, 1973), but AMP-aminohydrolase from human (Makarewicz & Stankiewicz, 1974) and chicken skeletal muscle (Henry & Chilson, 1973) show higher activity in the presence of K^+ ions than in the presence of ATP.

Data presented in this paper indicate that kinetics of the reaction catalysed by the purified AMP-aminohydrolase from human placenta are very similar to that shown by the brain enzyme but distinct from that of the muscle AMP-aminohydrolase. It has been repeatedly reported that much greater similarities are observed among AMP-aminohydrolases from the same tissue of different animals than among enzymes from different tissues of the same animal. Muscle enzymes from different species have been investigated and their kinetic (Makarewicz, 1969; Zielke & Suelter, 1971a; Makarewicz & Stankiewicz, 1974) and immunological (Selig & Chilson, 1969) properties have been shown to be very similar. On the other hand, differences exist between human placental AMP-aminohydrolase and AMP-aminohydrolase from human skeletal muscle (Makarewicz & Stankiewicz, 1974) and erythrocyte (Askari & Franklin, 1965; Askari, 1966; Rao *et al.*, 1968). Analogous differences in kinetics of the reaction and properties of AMP-aminohydrolase have been shown with the chicken muscle, brain and erythrocyte enzymes (Henry & Chilson, 1969, 1973).

Placental AMP-aminohydrolase is inhibited by inorganic phosphate to a higher extent than the enzyme from other sources (Lee & Wang, 1968; Makarewicz & Stankiewicz, 1974). The inhibition is less pronounced in the presence of ATP, similarly as it has been reported for rat brain enzyme (Lee & Wang, 1968). As both ATP and inorganic phosphate exert their effect at low, physiological concentrations, they might be important in regulating placental AMP-aminohydrolase activity *in vivo*.

AMP-aminohydrolase from skeletal muscle has been shown to be a metalloenzyme containing zinc (Zielke, 1970; Zielke & Suelter, 1971b; Raggi *et al.*, 1971). No data are available on the role of metal in the activity of AMP-aminohydrolase from other sources. Inactivation by EDTA and recovery of the activity upon addition of some divalent cations in micromolar concentrations suggests the requirement of the placental AMP-aminohydrolase for metal ions.

Recently Ogasawara *et al.* (1972) reported that four molecular forms of rat brain AMP-aminohydrolase could be separated by chromatography on phosphocellulose. These forms shown different K_m values, from 5 mM to 42 mM, and differ in sensitivity to cations and nucleotides. The existence of two forms of AMP-aminohydrolase in calf muscle has also been reported (Birnbaum *et al.*, 1971). The properties of the placental enzyme seem to be similar to those of form IV from rat brain (Ogasawara *et al.*, 1972) which shows very low affinity towards substrate (K_m 42 mM). It may be supposed that the pattern of different molecular forms of AMP-aminohydrolase is specific for particular tissues, with one form possibly predominating.

So far, the physiological role of AMP-aminohydrolase has not been elucidated. Lowenstein (1972) suggests that in muscle and other tissues (liver, brain, kidney, ascites cells and erythrocytes), the reaction catalysed by AMP-aminohydrolase is a part of a purine nucleotide cycle involving AMP deamination and IMP reamination. No data are available at present on the occurrence in placental tissue of the remaining enzymes of the cycle, i.e. IMP : L-aspartate ligase (GDP) (EC 6.3.4.4) and adenylsuccinate AMP-lyase (EC 3.4.2.2).

The observed differences in the regulatory properties of AMP-aminohydrolase may reflect the different roles which AMP-aminohydrolase plays in cellular metabolism.

The author wishes to thank Prof. Dr. M. Żydowo for his interest in this work, and Mrs. Regina Kaczorowska for technical assistance.

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OCZYSZCZANIE I WŁASNOŚCI AMP-AMINOHYDROLAZY ŁOŻYSKA LUDZKIEGO

Streszczenie

1. AMP-aminohydrolazę z łożyska ludzkiego oczyszczono około 570-krotnie.
2. Oczyszczony enzym ulega inaktywacji po dializie wobec 1 mM-EDTA; reaktywacja następowała po dodaniu niektórych kationów dwuwartościowych: Zn^{2+}, Mn^{2+} i Co^{2+}.
3. Enzym jest aktywowany przez jony Li^{+} i ATP. Fosforan nieorganiczny wykazuje silne działanie inhibitorowe.
4. Własności kinetyczne i regulacyjne AMP-aminohydrolazy z łożyska ludzkiego są podobne do enzymu z mózgu, natomiast są wyraźnie różne od enzymu z mięśni szkieletowych.

Received 11 October, 1973.

ACTA BIOCHIMICA POLONICA

Vol. 21 1974 No. 2

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ACTIVATION OF PREEXISTING MESSENGER RNA IN DRY PEA EMBRYO AXES **

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The occurrence of preformed mRNA in quiescent pea seed embryo axes has been demonstrated. In the early stage of germination, when no RNA synthesis was observed, incorporation of amino acids occurred simultaneously with polysome formation. The preformed inactive mRNA was associated with the ribosomal fraction and could be activated by trypsin treatment, demonstrated by the *in vitro* formation of new active polysomes.

During the last decade, thorough investigations were carried out on the presence of mRNA in the form of ribonucleoprotein complexes, called informosomes or masked RNA, in the latent stage of development in various organisms. Such forms of mRNA can survive long periods of metabolic dormancy and may be involved in the translation-level control, in the transport of mRNA from the nucleus or in stabilization of the labile mRNA molecules (see Gross, 1968 and Spirin, 1969, for reviews). Activation of the stable form of mRNA during the developmental phase induces corresponding changes in capacity for protein synthesis and a change in polysome content. Monroy *et al.* (1965) studied the cellular mechanism whereby polysome formation is facilitated and have shown that trypsin treatment of ribosomes of unfertilized sea urchin eggs results in increased ribosomal activity. Similar activation by trypsin has been observed in HeLa cells at the stage of metaphase (Salb & Marcus, 1965), rat liver (Östner & Hultin, 1968) and in cotyledons of pea seeds (Jachymczyk *et al.*, 1971).

In the early phases of seed germination, imbibition of water initiated protein synthesis with a simultaneous rapid conversion of ribosome population into polyribosomes (Marcus & Feeley, 1964, 1965; Barker & Rieber, 1967; Jachymczyk &

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** This work was supported by the Polish Academy of Sciences within the project 09.3.1.

Cherry, 1968). This conversion took place in the absence of RNA transcription (Dure & Waters, 1965; Marcus & Feeley, 1966; Chen *et al.*, 1968; Jachymczyk *et al.*, 1971; Sieliwanowicz & Chmielewska, 1973).

The present report describes the experiments which showed that RNA extracted from ribosomal fraction of quiescent and germinating pea embryo axes had a similarly high template activity in stimulating [^{14}C]amino acid incorporation in a cell-free system. This endogenous messenger activity in dry, quiescent embryo axes could be released by treatment of ribosomes with trypsin.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade. ATP and creatine phosphate were purchased from Calbiochem (Los Angeles, Calif., U.S.A.), trypsin 2× cryst. (spec. act. 3.5 units/mg of protein), GTP, creatine phosphokinase and 2-mercaptoethanol were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), Difco protein hydrolysate (vitamin-free Casamino acids) from Difco Laboratories Inc. (Detroit, Mich., U.S.A.). Sucrose ultra pure from Schwarz-Mann Comp. (Orangeburg, N. Y., U.S.A.). [G-^{3}H]Uridine (spec. act. 4.6 Ci/mmol) and a mixture of ^{14}C-labelled amino acids of a specific activity 52 mCi/milliatom of carbon, were purchased from the Radiochemical Centre (Amersham, Bucks., England).

Pea seeds (*Pisum sativum* L.) were germinated on moist paper in constant humidity at 25°C in a dark chamber. Embryo axes were excised manually both from dry and germinated seeds.

Sucrose density gradient analysis of ribosomes. The ribosomes were prepared according to the method of Davies *et al.* (1972) with the modification described below. Embryo axes, a hundred 10-mm segments weighing 1.5 - 2 g, isolated from the seeds germinated for the indicated period of time, or 600 mg of embryos from dry seeds were washed in water and incubated in a small volume (3 - 4 ml) of a solution containing 1% sucrose, 0.1 mM-ammonium citrate (pH 6), 20 μCi of [^{14}C]amino acids mixture and 25 μCi of [^{3}H]uridine. After 2 h incubation, the embryo axes were washed twice with cold water, frozen on solid CO_2 and ground in a mortar in 10 vol. of grinding buffer (0.4 M-sucrose, 20 mM-KCl, 5 mM-$MgCl_2$, 5 mM-2--mercaptoethanol and 100 mM-Tris-HCl buffer, pH 8.5). The resulting homogenate was clarified by centrifugation for 20 min at 20 000 *g* and the supernatant was gently layered over 4 ml of 1.5 M-sucrose in buffer *A* (40 mM-Tris-HCl, pH 8.5, 10 mM-$MgCl_2$, 20 mM-KCl) and centrifuged for 2 h at 105 000 *g* in the titanium angle rotor of a MSE superspeed 65 ultracentrifuge (10 × 10 ml). The pellets were rinsed and resuspended in 1 ml of buffer *A*. Samples (usually 1 mg in 0.5 ml) of resuspended ribosomes were layered on a linear sucrose gradient (125 - 500 mg/ml) prepared in buffer *B* (50 mM-Tris-HCl, pH 8.5, 10 mM-$MgCl_2$, 20 mM-KCl) and centrifuged for 150 min at 75 000 *g* in a MSE aluminium swing-out rotor (6 × 15 ml). To determine the distribution of ribosomes in the gradient, 40 - 45 fractions, 15 drops each, were collected from the bottom of the tubes. Samples were adjusted with water to a volume of 1 ml and extinction at 260 nm was measured in Unicam SP-500

spectrophotometer. Radioactivity of the double-labelled ribosomes were measured in each fraction as described by Jachymczyk & Cherry (1968).

Incorporation of ^{14}C*-labelled amino acids* in vitro. The ribosomes were isolated and the incorporation assayed under essentially the same conditions as those used by Jachymczyk & Cherry (1968) except that the "pH 5 enzymes" supplemented with [^{14}C]aminoacyl transfer RNA prepared according to Moldave (1963) were used instead of the 105 000 *g* supernatant.

The mixture of purified tRNA from wheat embryo free from amino acids, used for loading, was a kind gift of Dr. A. Legocki (Dept. of Biochemistry, Academy of Agriculture, Poznań).

The amino acid incorporation was stopped by placing a 100 µl portion of the incubation mixture on a Whatman 3 *MM* filter paper disc, drying in a stream of air and immersing in cold 10% trichloroacetic acid solution containing about 2% Difco casamino acids. On the next day, the samples were further treated according to Mans & Novelli (1961).

Radioactivity was assayed by means of a Packard Liquid Scintillation Spectrometer Tricarb model 3320. Total protein was determined by the method of Lowry *et al.* (1951).

Treatment of ribosomes with trypsin. Ribosomes were prepared as described earlier (Jachymczyk & Cherry, 1968) and samples (2 - 5 mg) were incubated at 37°C with trypsin (0.6 µg/1 mg of ribosomes in 1 ml). After 5 min the reaction mixture was cooled to 0°C, diluted with ice-cold grinding buffer to a volume of 8 ml and immediately centrifuged for 60 min at 105 000 *g*. The ribosomal pellets were washed twice with 0.2 M-sucrose in 5 mM-Tris-HCl buffer, pH 7.8, containing 10 mM-$MgCl_2$, 20 mM-KCl and 5 mM-2-mercaptoethanol, suspended in 0.2 ml of the same medium and used for the amino acid incorporation assays. After incubation, the samples were cooled rapidly to 0°C, layered over a linear sucrose gradient (125 - 500 mg/ml), centrifuged, and ribosome distribution was determined as described above.

RESULTS

Estimation of polyribosomes and of the protein and RNA synthesis in vivo. The results of sucrose gradient analysis presented in Fig. 1 illustrate the formation of polysomes and the incorporation of [^{14}C]amino acids and [^{3}H]uridine into the ribosomal fraction during the early stage of pea seed germination. In seeds germinated for 20 and 40 h, the formation of polyribosomes corresponded closely to binding of ^{14}C-labelled amino acids. However, there was practically no incorporation of [^{3}H]uridine either in dry seeds or in those germinated for 20 h. Incorporation of [^{3}H]uridine appeared only after 40 h of germination, the radioactivity being then present both in the polyribosome and monoribosome region. Thus it appears that the early formation of polysomes was dependent on the preformed messenger RNA present in dry seeds.

Amino acid incorporation in vitro. To confirm the presence of mRNA in ribosomes of dry pea embryo axes, the template activity of RNA isolated by the phenol-

-cresol method (Sieliwanowicz *et al.*, 1974) from the ribosomes of both dry and germinated seeds, were studied. It was found (Table 1) that the addition of RNA from ribosomes of dry embryo axes to a cell-free protein-synthesizing system containing ribosomes from embryos of dry seeds or seeds germinated for 40 h, resulted in,

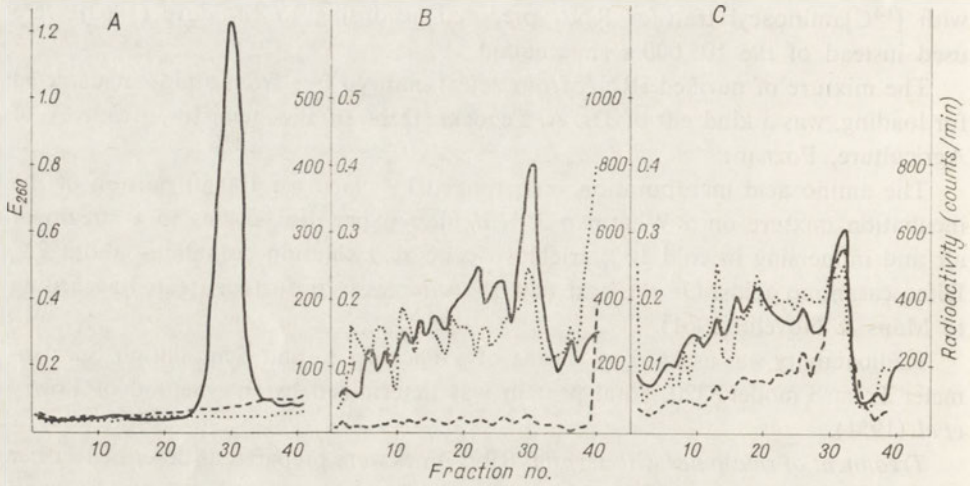

Fig. 1. Sucrose gradient profiles of ribosome preparations from embryo axes of pea seeds: *A*, dry; *B*, germinated for 20 h and *C*, germinated for 40 h. ——, E_{260}; - - -, radioactivity of [^{3}H]uridine; ···, radioactivity of [^{14}C]amino acids.

Table 1

The in vitro *activity of RNA isolated from ribosomal fraction of dry and germinated pea embryo axes*

Total RNA was isolated by the phenol-cresol method (Sieliwanowicz *et al.*, 1974). The complete system contained in a volume of 1 ml: 0.9 mg of ribosomes from dry embryo axes or 2 mg of ribosomes from germinated embryo axes, 0.7 mg protein of "pH 5 enzymes", 0.5 μCi of the mixture of ^{14}C-labelled amino acids, and, as messenger, 170 μg of RNA from the indicated material. The incubation was carried out at 37°C for 30 min.

Source of ribosomes in the cell-free system	Added RNA isolated from embryo axes	Incorporation (c.p.m./mg ribosomes)
Dry embryo axes	None (control)	344
	dry	2688
	germinated for 24 h	2083
	germinated for 48 h	2365
	postmicrosomal supernatant	427
Embryo axes germinated for 40 h	None (control)	3065
	dry	8290
	germinated for 24 h	6961
	germinated for 48 h	7598
	postmicrosomal supernatant	3317

respectively, sevenfold and twofold stimulation of the *in vitro* [^{14}C]amino acid incorporation. Practically the same stimulation of protein synthesis was observed with RNA isolated from ribosomes of germinated embryo axes. On the other hand, the RNA isolated from the postmicrosomal supernatant had no effect on the amino acid incorporation.

These results indicate that RNA isolated from the ribosomal fraction of dry pea embryo axes contained a messenger RNA. This preformed mRNA either could be bound to ribosomes or, what seems more probable, exists as distinct nucleoprotein particles, as it was found for animal tissues (Henshaw, 1968; Spirin, 1969) and wheat embryos (Weeks & Marcus, 1971). In this case, however, the sedimentation coefficient of such particles should be close to that of ribosomes.

Polysome formation in vitro. Ribosomes isolated from dry embryo axes were treated with a small amount of trypsin, then carefully washed to remove any traces of trypsin, and pulse-labelled with radioactive amino acids. When the incubation mixture was examined on the sucrose gradient (Fig. 2), the distribution of radio-

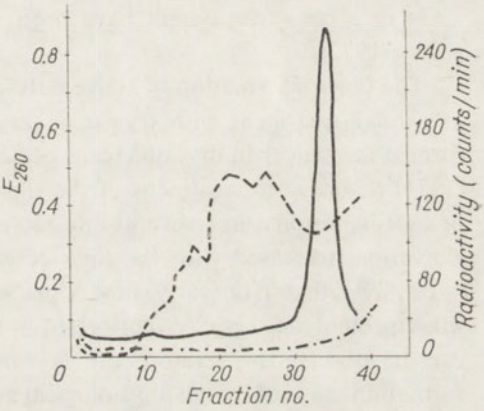

Fig. 2. Amino acid incorporating capacity of ribosomal preparation from quiescent pea embryo axes treated with trypsin. The amino acid incorporation medium was composed of 0.6 mg of ribosomes (treated or untreated with trypsin), 0.7 mg protein of "pH 5 enzymes" and other additions as in Jachymczyk & Cherry (1968) in a total volume of 0.4 ml. Samples were incubated for 5 min at 37°C. Radioactive amino acid pulse (0.5 μCi) was included for another 5 min before tayering on sucrose gradient. ——, E_{260} nm; - - -, radioactivity of ribosomes treated with lrypsin; ····, radioactivity of control ribosomes (untreated with trypsin).

activity differed from that of monoribosome absorbance. Since only polysomes catalyse incorporation, the observed incorporation of radioactivity indicated the occurrence of newly formed polysomes. In contrast, the ribosomal fraction untreated with trypsin (control experiment) remained completely inactive. Thus it seems that trypsin released the preexisting messenger from the complex with protein inhibitor. However, the susceptibility of ribosomal fraction to any excess of trypsin (Jachymczyk *et al.*, 1971) and relatively low ^{14}C incorporation suggests that also other protein components important for translation, may be destroyed.

DISCUSSION

Weeks & Marcus (1971) were the first to demonstrate directly the existence of mRNA in wheat embryo. This finding was confirmed by Schultz *et al.* (1972). The experiments described in the present work showed the presence of mRNA in dry pea embryo axes. The observed lack of [^{3}H]uridine incorporation during the early

stage of pea seed germination, despite the presence of enzymes necessary for RNA synthesis (Barker & Rieber, 1967), together with the simultaneous formation of polysomes and [^{14}C]amino acid incorporation, showed that, at the beginning of germination, translation precedes markedly the genomic transcription. Thus the required messenger activity must be provided by the mRNA preexisting in the quiescent pea seeds, and indeed the RNA isolated from ribosomal fraction of dry embryo axes was found to possess template activity. Sieliwanowicz *et al.* (1974) by acrylamide-gel electrophoresis of RNA isolated from ribosomal fraction of dry and germinating pea seeds demonstrated, in addition to the normally observed 24s, 17s and 5s ribosomal RNA species, the presence of some minor species between 17s and 5s. These latter RNA species were not observed in the RNA extracted from postmicrosomal supernatant which contained ribosomal subparticles and soluble RNA, and indeed showed no template activity in cell-free system. Therefore it may be supposed that the RNA responsible for template activity appears only in the fractions which on electrophoresis migrate between the 17s and 4 - 5s species.

Similar minor species active in stimulation of the incorporation of [^{14}C] amino acids in a cell - free system have been reported in dry wheat embryos by Schultz *et al.* (1972).

The *in vitro* formation of active polysomes in ribosomal fraction from dry embryo axes after treatment with trypsin is an additional proof for the existence of preformed messenger in dry, quiescent pea embryos. It was found by Jachymczyk *et al.* (1971) that trypsin treatment of the ribosomal fraction from the cotyledons of dry pea seeds resulted in a several-fold increase in their activity, and that the degree of activation decreased with the time of germination. Recently Hobday *et al.* (1973) have shown that in the early stage of pea seed germination, a significant rise in trypsin--like proteolytic activity, connected with a decrease in trypsin inhibitor activity, can be observed. Therefore the *in vitro* activation by trypsin of polyribosome formation may reflect the physiological activation of the preexisting messenger, which would consist in a release of mRNA from inactive complexes with protein inhibitor of translation.

The authors are deeply grateful to Professor Dr. Irena Chmielewska for her continued interest in this work and for comments on the manuscript. Thanks are also due to Dr. A. Legocki for the gift of highly purified tRNA and to Miss E. Kolanowska for help in obtaining RNA preparations.

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AKTYWACJA ZABLOKOWANEJ FORMY mRNA W OSIACH ZARODKOWYCH SUCHYCH NASION GROCHU

Streszczenie

Wykazano obecność zablokowanej formy mRNA w osiach zarodkowych suchych nasion grochu. We wczesnych stadiach kiełkowania włączanie radioaktywnych aminokwasów połączone z powstawaniem polisomów znacznie wyprzedza w czasie syntezę nowych cząstek RNA. Stwierdzono, że zablokowana, nieaktywna forma mRNA towarzyszy frakcji rybosomalnej i może być aktywowana przez działanie trypsyną, co powoduje tworzenie się *in vitro* polisomów, zdolnych do włączania radioaktywnych aminokwasów do białek.

Received 17 October, 1973.

ACTA BIOCHIMICA POLONICA

Vol. 21 1974 No. 2

MAGDALENA FIKUS

A SIMPLE ENZYMIC SYNTHESIS OF 5′-TERMINAL INOSINE-CONTAINING DINUCLEOSIDES *

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1. 5′-Terminal inosine-containing dinucleosides have been synthesized using RNase T_1 in a way analogous to that described recently for 5′-terminal xanthosine-dinucleoside monophosphate (M. Fikus, *Acta Biochim. Polon.*, 1973, **20**, 237 - 248). Several dinucleosides containing 3′-terminal pyrimidines with variously modified base and/or sugar were obtained in 30 - 40% yield. 2. The reaction products were characterized by their chromatographic properties and u.v. spectra. 3. In the same way products of enzymic and chemical hydrolysis of the dinucleosides were analysed.

With the progress of investigations on the structure and conformation of synthetic polynucleotides and nucleic acids, a need has arisen for the development of simple and efficient methods of synthesis of model compounds, such as oligonucleotides of known sequence and chain length.

The present communication is a sequel to a previous publication (Fikus, 1973) and deals with the possibility of using RNase T_1 for the synthesis of dinucleoside monophosphates containing 5′-terminal inosine.

METHODS

Inosine 2′(3′)phosphate was obtained by deamination of the corresponding adenosine 2′(3′)phosphate under conditions described by Shapiro & Pohl (1968), used previously for deamination of guanosine phosphate (Fikus, 1973). The deamination product was purified according to Ganguli *et al.* (1971). The fractions containing inosine phosphate were neutralized with ammonia, evaporated and extracted with anhydrous methanol.

* This work was supported by the Polish Academy of Sciences within the project 09.3.1, and also profited from the support of The Wellcome Trust and the World Health Organization.

Inosine 2′,3′-cyclic phosphate was prepared in 100% yield according to Shugar (1967). All the measurement techniques, methods and other compounds used, were as described previously (Fikus, 1973). The incubation mixture contained 10 - 80 mM-inosine 2′,3′-cyclic phosphate, 0.1 - 1 M-nucleosides, and RNase T_1 at concentration of 35 - 100 u./ml in 0.1 M-cacodylate buffer. The yields were expressed as percentage of transformed inosine 2′,3′-cyclic phosphate. The reaction products were identified by their chromatographic R_F values as well as by u.v. spectra (Fig. 1).

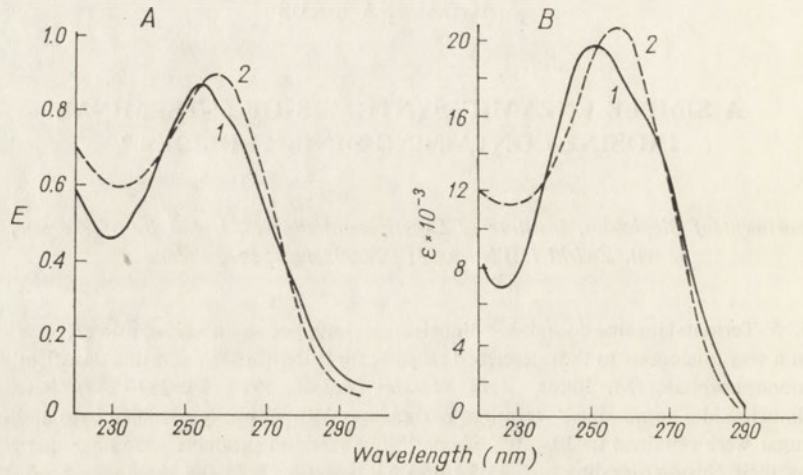

Fig. 1. U.v. spectra of inosylyl(3′-5′)uridine dinucleoside monophosphate synthesized by RNase T_1 from inosine cyclic phosphate and uridine (*A*), in comparison with the calculated spectra of an equimolar mixture of uridine and inosine (*B*). Spectra were taken in H_2O (*1*) and at pH 12 (*2*).

Dinucleoside monophosphates were hydrolysed in alkaline medium and the hydrolysates were quantitated by paper chromatography on Whatman no. 1 paper in ethanol - 1 M-ammonium acetate, pH 7.5 (7 : 3 by vol.) (solvent *A*). This permitted to prove composition and sequence of the monomers in the dinucleoside investigated (cf. Fikus, 1973).

RESULTS AND DISCUSSION

The yield of inosylyl(3′-5′)uridine dinucleoside monophosphate (IU) within the investigated pH range of 5.5 - 7.3 was maximal at pH 6.3. For example, after 3 h of synthesis, the yields were: 26, 33 and 23% at pH 5.6, 6.3 and 7.3, respectively. These results are in accordance with the optimum pH value for the hydrolysis of inosine 2′,3′-cyclic phosphate by RNase T_1 (Irie *et al.*, 1970).

Within the range of substrates and enzyme concentrations used, the yield of IU was maximal after 3 h incubation. Further incubation led to a gradual enzymic hydrolysis of cyclic phosphate and/or IU, and to the accumulation of inosine phosphate. At lower concentrations of substrates (10 mM-cyclic phosphate and 0.1 M-nucleoside) and the enzyme (35 u./ml) dinucleosides were obtained in a 25%

yield whereas at higher concentrations (80 mM-cyclic phosphate, 1 M-nucleoside and 100 u./ml of the enzyme) the yield was increased to 40%. The maximum yields of dinucleoside at 37° and 4°C were comparable, the only difference being that at 37°C the reactions, as would be expected, were faster.

The results of chromatographic analysis of the reaction products on t.l.c. in saturated ammonium sulfate - 1 M-ammonium acetate - isopropanol (80:18:2, by vol.) (solvent *B*) revealed that inosine 3′-phosphate was the only product following: (a) RNase T_1 action upon inosine 2′,3′-cyclic phosphate (20 mM-inosine 2′,3′-cyclic phosphate, RNase T_1 350 u./ml, pH 6.3, 48 h at 37°C); (b) hydrolysis of IU by RNase T_1 (4.6 mM-IU, RNase T_1 350 u./ml, pH 6.3, 48 h at 37°C).

No inosine 2′-phosphate was found in either of the hydrolysates. Thus we conclude that RNase T_1 synthesized specifically the phosphodiester bond of the inosylyl (3′-5′)uridine type.

Syntheses of dinucleoside monophosphates were attempted under the conditions optimal for IU formation, using as substrates the following nucleosides: cytidine, 6-methylcytidine, 1-β-D-arabinofuranosylcytosine, 2′,3′-*O*-dimethylcytidine, 2′,3′-*O*-dimethyluridine and 2′-*O*-methylcytidine. In all cases the products obtained were identified chromatographically and spectrally as dinucleoside monophosphates (Table 1). The yields of these reactions were approximately the same (30%).

It was found that xanthosine could not serve as the nucleoside substrate.

Attempts were also made to synthesize homodinucleoside mono- and diphosphates starting with inosine 2′,3′-cyclic phosphate or inosine 2′,3′-cyclic phosphate plus inosine. In the first case the concentration of inosine 2′,3′-cyclic phosphate was 0.4 M, and that of RNase T_1 110 u./ml (pH 6.3, 72 h at 37°C). Progress of the reaction

Table 1

R_F values of nucleoside substrates and of the enzymically synthesized dinucleoside monophosphates containing 5′-terminal inosine

Conditions: solvent *A*, Whatman no. 1 paper. In this system the R_F value for inosine 2′,3′-cyclic phosphate was 0.55, and for inosine 2′(3′)phosphate 0.18.

3′-Terminal nucleoside substrate	R_F	Corresponding dinucleoside monophosphate product	R_F
Uridine	0.72		0.42
2′-*O*-Methyluridine	0.82		0.48
2′,3′-*O*-Dimethyluridine	0.79		0.51
Cytidine	0.65		0.51
2′,3′-*O*-Dimethylcytidine	0.82		0.69
6-Methylcytidine	0.80		0.39
1-β-D-Arabinofuranosylcytosine	0.76		0.35
Inosine	0.59		0.32

was controlled chromatographically. After 72 h the incubation mixture was diluted 15-fold with HCl solution to pH 1 and then kept at room temperature for 3 h (which is the time required for opening of the cyclic phosphate rings). Finally, the mixture was applied on the DEAE-cellulose column under conditions described previously (Fikus, 1973). Inosine 2′(3′)phosphate was eluted with 0.11 M, and the dinucleotide with 0.28 M-triethylamine bicarbonate. Very low yield of the dinucleotide (2%) proved the inefficiency of this method of preparation.

The second procedure for the synthesis of homodinucleoside monophosphate consisted in incubating inosine 2′,3′-cyclic phosphate (40 mM) with saturated inosine solution at pH 6.3 in the presence of 35 units of RNase T_1 per 1 ml. T.l.c. of the incubation mixture in solvent *A*, after 48 h incubation, revealed the presence of a product which, upon alkaline hydrolysis, gave inosine 2′(3′)phosphate and inosine. This supports our conclusion that the enzymically synthesized product was inosylyl(3′--5′)inosine. The yield of reaction in this case was also low and did not exceeded 10%.

To sum up, RNase T_1 is suitable for the synthesis of dinucleoside monophosphates containing 5′-terminal inosine and 3′-terminal pyrimidine nucleoside, irrespective of the differences in the base or sugar moieties. The yield was 30 - 40% of the amount of the substrate used, i.e. inosine 2′,3′-cyclic phosphate. All the reactions and controls are easy to perform even in a laboratory without special equipment.

The results reported on these model reactions permit a full comparison of the synthetic activity of RNase T_1 towards 2′,3′-cyclic phosphates of guanosine, xanthosine and inosine as they are found during the enzymic (by RNase T_1) hydrolysis of the native and deaminated RNA's.

The common trait of all these synthetic reactions is the formation of dinucleoside monophosphates with the purine nucleoside in the 5′terminal position. The amounts of the components of the incubation mixture providing for the optimal yield are closely similar: cyclic phosphates, 10 - 100 mM; 3′-terminal nucleosides, 0.1 - 1 M; RNase T_1, 30 - 500 u./ml. In all cases the investigated pyrimidine derivatives modified to various extent in their base and sugar rings were incorporated easily. Dinucleosides with 3′-terminal purine nucleoside were synthesized rarely and/or in low yield, which could not be accounted for solely by low solubility of purine nucleosides (Mohr & Thach, 1969). The yields of the dinucleosides are closely comparable and reasonably high under appropriate incubation conditions (Mohr & Thach, 1969; Rowe & Smith, 1970; Fikus, 1973).

The optimum pH for the synthetic reaction depends on the cyclic phosphate used as the substrate. In the case of the reaction with guanosine, xanthosine and inosine the optimum pH is 7.5, 5.5 and 6.3, respectively. Irie *et al.* (1970) relate these findings to the differences in the p*K* values for ionization of the N_1 hydrogens of the substrates used.

The synthesis of dinucleoside monophosphates from guanosine cyclic phosphate is more efficient at low (0°C) temperature (Rowe & Smith, 1970), whereas the yield of the reaction with xanthosine and inosine cyclic phosphates is the same at 0 and 37°C. This is probably due to the differences in susceptibility of the corresponding dinucleosides to hydrolysis by RNase T_1 (Witfeld & Witzel, 1963).

Finally, homooligonucleotides are formed enzymically from concentrated (up to 1 M) solutions of cyclic nucleotides with varying efficiency, which decreases in the order: guanosine, xanthosine, inosine cyclic phosphates (Sekiya *et al.*, 1968; Fikus, 1973). These differences may perhaps be due in part to the ability of these monomers to form stacked aggregates in concentrated aqueous solutions (Raszka & Kaplan, 1972).

I am greatly indebted to Professor D. Shugar for his interest in these investigations; to Dr. J. Kuśmierek for a gift of 1-β-D-arabinofuranosylcytosine, 2′,3′-*O*-dimethylcytidine, 2′-*O*-methyl-, 2′,3′-dimethyluridines; to Dr. M. Świerkowski for a sample of 6-methylcytidine, and to Mrs. D. Haber for her skilfull technical assistance.

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ENZYMATYCZNA SYNTEZA DWUNUKLEOZYDOMONOFOSFORANÓW ZAWIERAJĄCYCH 5′-TERMINALNĄ INOZYNĘ

Streszczenie

Praca jest kontynuacją opisanej uprzednio metody enzymatycznej syntezy dwunukleozydomonofosforanów zawierających 5′-terminalną ksantozynę (Fikus, 1973). Stosując analogiczne metody syntez i analizy produktów otrzymano z 30 - 40% wydajnością dwunukleozydomonofosforany zawierające 5′-terminalną inozynę i szereg 3′-terminalnych pochodnych pirymidynowych o różnym stopniu modyfikacji pierścieni cukru i/lub zasady. Produkty syntezy charakteryzowano na podstawie ich cech chromatograficznych oraz widm w nadfiolecie; analizowano również produkty ich enzymatycznej i chemicznej hydrolizy.

Received 22 November, 1973.

ACTA BIOCHIMICA POLONICA
Vol. 21 1974 No. 2

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INTERACTION WITH DNA OF THE ACETYLATED AND NON-ACETYLATED POLYVALENT BASIC TRYPSIN INHIBITOR OF THE KUNITZ TYPE *

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1. On the basis of optical rotatory dispersion, circular dichroism and i.r. spectra it has been demonstrated that acetylation of ε-amino lysine residues of the basic polyvalent trypsin inhibitor leads to changes in its secondary structure, the content of β-structure being increased and that of α-helix unchanged. Simultaneously the inhibitor becomes inactive towards trypsin, retaining its activity towards chymotrypsin. 2. The behaviour of soluble complexes of DNA with the acetylated and non-acetylated inhibitor was studied. From the u.v. difference spectra and melting profiles it follows that the native inhibitor stabilizes the double helix of DNA, whereas the acetylated preparation has a destabilizing effect.

In the previous work (Szopa, 1974) it has been demonstrated that the complexes of DNA with acetylated histones have a considerably lower stability than those with non-acetylated histones. Moreover, it was found that the acetylated histones have a more ordered structure as compared with the non-acetylated ones.

To obtain more information on the role of basic proteins in chromatin, complexes of DNA with another acetylated and non-acetylated basic protein were investigated. For this purpose, the basic polyvalent trypsin inhibitor of the Kunitz type was chosen. This inhibitor is a protein of known molecular weight (6500) and known amino acid sequence (Vogel *et al.*, 1968).

MATERIALS AND METHODS

Preparation of DNA, u.v. difference spectra, i.r. spectra, estimation of T_m values, and determinations of protein, DNA and phosphorus were performed as described previously (Szopa, 1974).

Optical rotatory dispersion (o.r.d.) and circular dichroism (c.d.) spectra were obtained in a Jasco model ORD UV-5 spectropolarimeter.

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

The polyvalent basic trypsin inhibitor of the Kunitz type was prepared from bovine lung as described by Wilusz *et al.* (1973). Its activity against trypsin and chymotrypsin was determined according to Kunitz (1947) using casein as substrate. Trypsin (1 × cryst.) was from Koch-Light Lab. Ltd (Colnbrook, Bucks., England), and chymotrypsin (3 × cryst.) from Reanal (Budapest, Hungary).

The inhibitor preparation was acetylated by the method described by Thomas *et al.* (1968), in which only the free ε-amino groups of lysine undergo acetylation. When tested by the methods of Habeeb (1966) and Nohara *et al.* (1968), the acetylated preparation showed the absence of free ε-amino groups or labile acetyl groups, respectively.

The inhibitor, non-acetylated or acetylated, was dissolved in 0.01 M-NaCl, pH 6.9, to a concentration of 150 μg/ml, and diluted with 0.01 M-NaCl to the required concentration.

To obtain soluble complexes of DNA with the inhibitor, DNA solution in 0.15 M-NaCl - 0.015 M-sodium citrate was dialysed against 0.01 M-NaCl, pH 6.9, and diluted to a concentration of 40 μg/ml; to 3 ml of the DNA solution was added an equal volume of the solution of non-acetylated or acetylated inhibitor, of such a concentration as to obtain the required inhibitor to DNA ratio. The samples were filtered through a sintered-glass filter G-4. The extinction at 340 and 400 nm of solutions of the complexes did not exceed that of free DNA (20 μg/ml) by more than 0.005 unit.

To study precipitation of DNA with the inhibitor, to 2 ml of the solution containing 40 μg DNA per 1 ml, was added an equal volume of the inhibitor solution of appropriate concentration. The sample was stirred for 10 min, then centrifuged in a Unipan type 310 centrifuge for 40 min at 14 000 rev./min at 3°C.

RESULTS AND DISCUSSION

Effect of acetylation on the properties of the trypsin inhibitor. The non-acetylated inhibitor preparation was homogeneous on polyacrylamide-gel electrophoresis under conditions described by Panyim & Chalkley (1969), whereas after acetylation it migrated slower, was less intensely stained, and resolved into several closely migrating fractions, two of which were more deeply stained with Amido Black.

The acetylated inhibitor lost the ability to inhibit trypsin but retained 90 - 95% of the activity towards chymotrypsin.

From the o.r.d. spectra presented in Fig. 1 it may be seen that the spectrum of the non-acetylated inhibitor has a trough at 227 nm, and that of the acetylated inhibitor, at 226 nm. The calculated Moffitt-Yang parameters (cf. Squire & Bewley, 1965) for the non-acetylated inhibitor preparation are: $a_0 = -620$, $b_0 = -114$ deg cm^2 d mol^{-1}, and for the acetylated one, $a_0 = -600$, $b_0 = -107$ deg cm^2 d mol^{-1}. The b_0 values indicate that the non-acetylated inhibitor contains 18% of α-helix, and the acetylated preparation, 17%. In both cases high negative values of a_0 indicate the prevalence of the random conformation over the other ones, especially pointing to low content of β-structure.

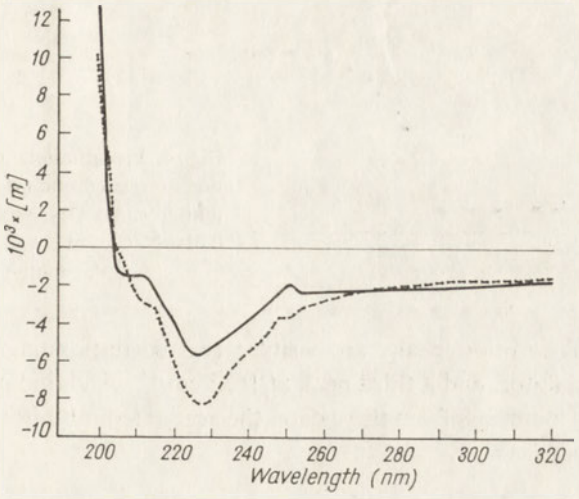

Fig. 1. Optical rotatory dispersion spectra of (- - -), non-acetylated and (——), acetylated trypsin inhibitor. The concentration of inhibitor was 20 μg/ml of 0.01 M-NaCl solution, pH 6.9.

The values of Θ at 222 nm in the c.d. spectrum of the two preparations of the inhibitor (Fig. 2) support the above conclusions concerning the content of α-helix.

The o.r.d. and c.d. spectra do not form an adequate basis for estimating the content of β-structure. The most suitable method for β-structure determination in polypeptides is the analysis of the i.r. spectrum in the region of amide I band. In the spectrum of the non-acetylated inhibitor in this region (Fig. 3), two absorption peaks may be discerned: at 1655 cm^{-1} pointing to the presence of α-helix, and at 1645 cm^{-1} indicating the presence of random conformation; the peak corresponding to β-structure being absent. In the same range of the spectrum of the acetylated

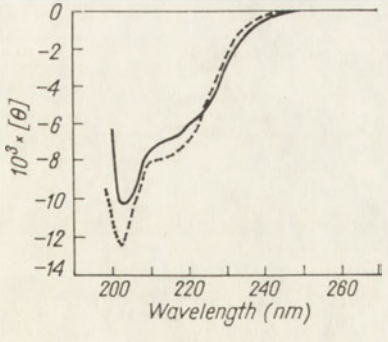

Fig. 2

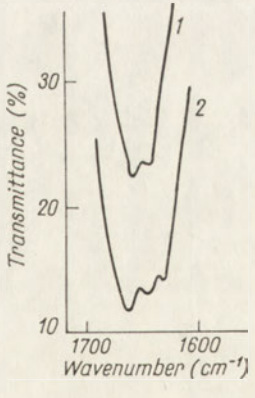

Fig. 3

Fig. 2. Circular dichroism spectra of (- - -), non-acetylated and (——), acetylated trypsin inhibitor. The concentration of inhibitor was 20 μg/ml of 0.01 M-NaCl solution, pH 6.9.

Fig. 3. Infrared spectra of *1*, non-acetylated and *2*, acetylated trypsin inhibitor in solid state.

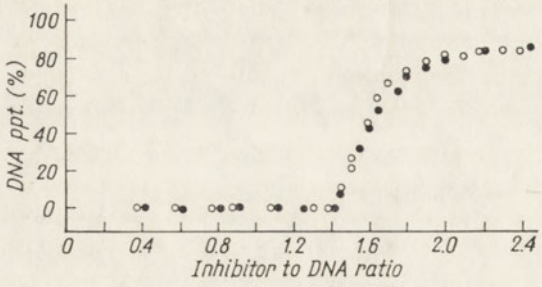

Fig. 4. Precipitation of DNA by (○), non-acetylated and (●), acetylated trypsin inhibitor. To DNA solution (20 μg/ml of 0.01M-NaCl, pH 6.9), different amounts of the inhibitor were added.

inhibitor, three absorption peaks are visible: two corresponding to those of the non-acetylated inhibitor, and a third peak at 1633 cm^{-1} which indicates the presence of β-structure. As it appears from these data, the acetylated inhibitor has an increased content of β-structure.

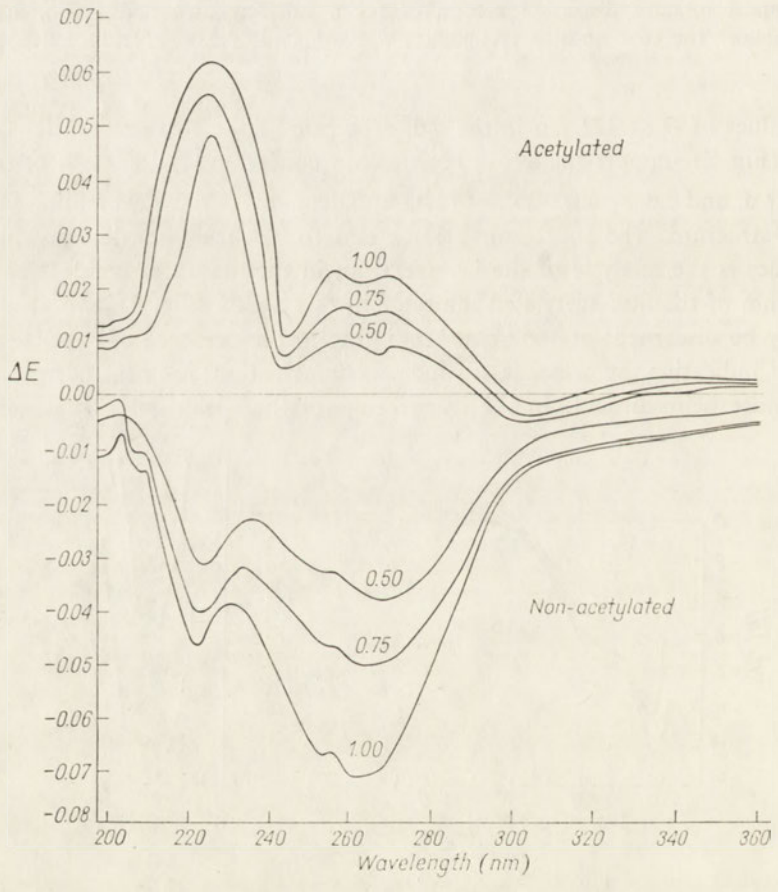

Fig. 5. U.v. difference spectra of DNA complexes with the acetylated and non-acetylated trypsin inhibitor, at the indicated inhibitor to DNA ratios. The final amount of DNA in the sample was 20 μg/ml.

Complexes of DNA with the non-acetylated and acetylated trypsin inhibitor. Dependence of DNA precipitation on the inhibitor to DNA ratio was practically the same with the acetylated and non-acetylated preparations, in both cases the maximum precipitation being obtained at the weight ratio of 2.0 (Fig. 4).

The u.v. difference spectra of the complexes over the range 200 - 340 nm (Fig. 5) show that with an increase in the non-acetylated inhibitor to DNA ratio, the minimum is shifted from 268 to 260 nm, indicating stabilization of the double helix of DNA by the inhibitor. The spectra of the complexes with the acetylated inhibitor show minima at about 298 nm and broad maxima over the region 250 - 270 nm. The complexes showing such spectra should have a DNA double helix less stable than the complexes with the non-acetylated inhibitor.

From the u.v. difference spectra it could be anticipated that the DNA in complexes with the non-acetylated inhibitor would show T_m values higher than those for free DNA. From the data presented in Fig. 6 and Table 1 it may be seen that this was indeed the case. With an increase in the inhibitor to DNA ratio, both the T_m values and hyperchroism slightly decreased. The hypochroism of the inhibitor-DNA complexes appearing at about 60°C is difficult to interpret. A similar phenomenon has been observed by Bobb (1968) for DNA-chymotrypsinogen complexes at about 60°C, who interpreted it as being due to release of protein. If this were so, the E_{260}/E_{240} ratio (which gives information on the amount of bound protein) should increase. However, as it is shown in Fig. 6C, this ratio decreased in the temperature range at which the hypochroism was observed.

The melting profiles of the acetylated inhibitor-DNA complexes at the ratios of 0.5 and 0.75 show a biphasic character. The first broad transition is observed at temperatures much lower than the T_m of free DNA, whereas the second transition appears at a higher temperature. This second transition was not observed for the DNA complexes with the non-acetylated inhibitor, and it seems possible that it

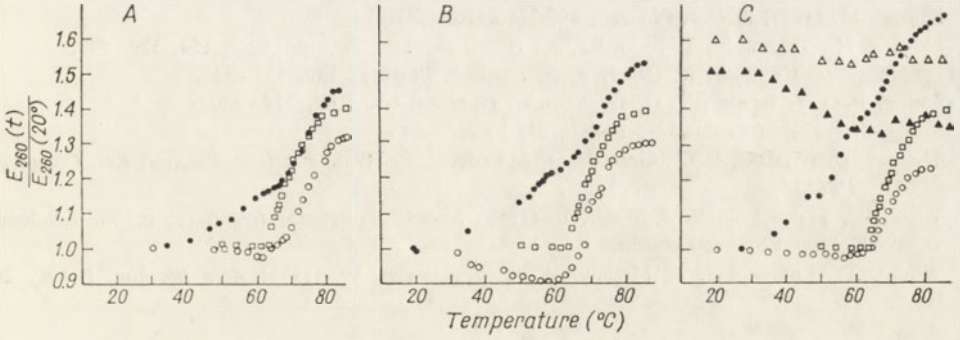

Fig. 6. Melting profiles of: (□), free DNA, and DNA complexes with (○), non-acetylated and (●) acetylated trypsin inhibitor, at the inhibitor to DNA ratios of: *A*, 0.50; *B*, 0.75; *C*, 1.00. In Fig. 6C the E_{260}/E_{240} ratios are also given for the complexes with (△) non-acetylated and (▲) acetylated inhibitor

could appear only at temperatures exceeding those studied in the present experiments.

At the 1.0 ratio of the acetylated inhibitor to DNA, the melting profile of the complex has a single-phase character, the T_m remaining much lower than for free DNA. These effects could have resulted either from a change in turbidity, release of protein from the complex, or destabilization of the DNA double helix by the acetylated inhibitor. The first possibility may be excluded as the turbidity remained unchanged over the studied temperature range. The second possibility may also be excluded because the E_{260}/E_{240} ratio decreased with the increase in temperature. Thus there remains only the last possibility, namely the destabilization of the native DNA structure.

In the previous work (Szopa, 1974) it was found that complexes of DNA with acetylated histones show somewhat lower stability than complexes with non-acetylated histones. However, the T_m of DNA complexes with acetylated histones were higher than that for free DNA, which could be interpreted as stabilization of the DNA double helix.

The u.v. difference spectra and melting profiles obtained in the present work indicate that the non-acetylated polyvalent basic trypsin inhibitor stabilizes, as anticipated, the double helix of DNA, whereas the acetylated inhibitor distinctly destabilizes DNA.

The author wishes to express his gratitude to Prof. Dr. Wanda Mejbaum-Katzenellenbogen for her interest in this work and helpful comments on the manuscript.

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INTERAKCJA Z DNA POLIWALENTNEGO ZASADOWEGO INHIBITORA TRYPSYNY TYPU KUNITZA PRZED I PO ACETYLACJI

Streszczenie

1. Na podstawie widm ORD, CD i IR wykazano, że acetylacja ε-aminowych reszt lizyny inhibitora trypsyny powoduje zmiany w jego strukturze drugorzędowej: zwiększa się zawartość β-struktury przy niezmienionej zawartości α-spirali. Inhibitor traci aktywność antytrypsynową, a zachowuje aktywność antychymotrypsynową.

2. Zbadano zachowanie się rozpuszczalnych kompleksów DNA z inhibitorem przed i po acetylacji. Z widm UV i oznaczenia profilów topnienia wynika, że inhibitor stabilizuje, a po acetylacji destabilizuje, podwójny heliks DNA.

Received 14 November, 1973.

ACTA BIOCHIMICA POLONICA

Vol. 21 1974 No. 2

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ON THE HORMONAL MODULATION OF ACUTE-PHASE PLASMA PROTEIN SYNTHESIS IN PERFUSED RAT LIVER *

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1. Livers from normal or turpentine-injected rats were perfused for 4 h with reconstituted blood enriched in amino acids, and incorporation of [^{3}H]lysine into plasma albumin, fibrinogen, ceruloplasmin, seromucoid fraction and total protein was determined. 2. Injection of turpentine stimulated the synthesis of fibrinogen, seromucoid and total protein while the radioactivity in albumin was slightly reduced. 3. The addition of cortisol, insulin and growth hormone to the reconstituted blood stimulated somewhat the acute-phase response. It is concluded that the hormones only modulate the rate of trauma-induced synthesis of plasma glycoproteins while the primary stimulatory factors originate probably from the site of injury.

The mechanism of trauma-induced synthesis of certain plasma glycoproteins (acute-phase reactants) in liver is still not completely elucidated. It has been suggested that humoral factors released in the site of injury, probably with the involvement of lysosomal enzymes, stimulate liver cells to enhanced formation of acute-phase reactants (Koj, 1970a). John & Miller (1969) reported the induction of synthesis of fibrinogen, α_1-acid glycoprotein, α_2-(acute-phase) globulin and haptoglobin by amino acids and hormones (insulin, cortisol and growth hormone) during prolonged perfusions of isolated normal rat livers. Since conflicting reports exist on the effect of administration of hormones on the acute-phase response (cf. Koj, 1974) we carried out experiments on this line with the perfused livers from control rats or from those with local inflammation evoked by a subcutaneous injection of turpentine oil.

MATERIALS AND METHODS

Liver perfusion. Male Wistar rats (300 - 400 g body weight) maintained on a standard pelleted diet were allowed food and tap water *ad libitum*. Some of them were

* This work was partially supported by a grant (202/II) from the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

injected subcutaneously with 0.5 ml turpentine oil (pharmaceutical preparation) 20 h before the experiment. This interval was selected as corresponding to the maximum acute-phase protein response to the phlogogenic stimulus (Koj, 1970b). Livers were isolated, and perfused with reconstituted blood according to John & Miller (1969) using the apparatus and technique described by Cohen & Gordon (1958). After cannulation the liver was briefly perfused with heparinized saline in order to remove the residual plasma, then perfused with the reconstituted blood consisting of 38 ml washed bovine red cells, 60 ml of Krebs-Ringer bicarbonate solution containing 3 g human serum albumin, 60 mg glucose, 1 ml (5000 units) of heparin, 3000 units of penicillin and 3 mg of streptomycin. In experiments on the effect of hormones, the reconstituted blood was enriched with 5 mg of cortisol, 6 units of insulin, and 2 units of bovine growth hormone, the employed amounts of hormones being suggested as the optimal in the experiments of John & Miller (1969). The volume of the reconstituted blood circulating in the perfusion apparatus was between 60 and 70 ml, and the flow rate through the liver was approximately 10 ml/min. The experiment was started by the addition of 5 μCi [^{3}H]lysine in 1.5 ml of Krebs-Ringer bicarbonate solution containing 30 mg of glucose and 25 mg of the amino acid mixture prepared according to John & Miller (1969) but without unlabelled lysine. An identical dose was given again 1 h later. In the experiments with reconstituted blood enriched in hormones they were also added to the amino acid mixture, the total amount in two supplementary doses being as follows: cortisol — 1 mg, insulin — 2 units, growth hormone — 1 unit.

The perfusion was continued for 4 h, pH being controlled every 20 min with a Ridan pH-meter. The decrease in pH observed during the experiment (especially in the presence of hormones) and reported by Lueck & Miller (1970) was corrected by the addition of isotonic $NaHCO_3$ solution to maintain the pH value between 7.3 and 7.4.

By the end of perfusion the circulating blood was collected and the liver was weighed. The plasma was obtained by centrifugation and dialysed twice for 24 h against 2 × 1 litre of citrate-saline solution (800 ml 0.9% NaCl + 200 ml 3.8% trisodium citrate) containing carrier unlabelled lysine.

Isolation of proteins from the dialysed plasma. Albumin was isolated by the trichloroacetic acid (TCA) - ethanol method in the following modification: 5 mg of carrier human γ-globulin was dissolved in 1 ml of plasma followed by the addition of 9 ml 0.1% solution of freshly prepared TCA in 96% ethanol. Without the addition of carrier γ-globulins no visible precipitate appeared under these conditions. After standing for 30 min at room temperature the sample was centrifuged for 5 min at 3000 r.p.m. and the supernatant was dialysed overnight against running tap water. The formed precipitate was removed by centrifugation and the supernatant was mixed with an equal volume of 10% TCA. The final precipitate was dissolved in 2 ml of 0.2 M-NaOH and samples were taken for the determination of radioactivity and protein content.

Fibrinogen was isolated as fibrin after clotting with thrombin: 10 mg of bovine carrier fibrinogen (obtained by the salting-out procedure described by McFarlane,

1963) in 5 ml of 0.9% NaCl was added to 5 ml of the plasma followed by 5.5 ml of saturated $(NH_4)_2SO_4$. After 1 h standing the sample was centrifuged for 5 min at 3000 r.p.m., the precipitate dissolved in 5 ml of citrate buffer, pH 6 (McFarlane, 1963), and dialysed overnight against 200 ml of this buffer. The solution was clarified by a brief centrifugation and diluted with 10 ml of water containing 1 mg of salmine sulphate. After addition of 10 units of thrombin the solution was mixed and then left for 2 h in a test tube with a glass rod immersed. The clot was collected onto the glass rod, washed by soaking in 0.9% NaCl (2 × 10 min) and dissolved in 3 ml of 0.2 M-NaOH by 4 min heating at 80°C. Samples of this solution were taken for the determination of radioactivity and protein.

Ceruloplasmin was isolated in the following way: 5 ml of plasma was mixed with 1 ml of carrier human ceruloplasmin containing 10 mg of protein. Five milliliters of this mixture was applied to a DEAE-Sephadex column (0.5 × 2 cm) and ceruloplasmin was isolated by the method of Stokes (1967). Losses of ceruloplasmin in this procedure were accurately determined by an enzymic method (Ravin, 1961). The obtained preparation was contaminated mainly with albumin, most of which could be removed by a procedure based on the TCA-acetone method of Schwert (1957): the solution of ceruloplasmin obtained from DEAE-Sephadex column was mixed with an equal volume of 10% TCA and centrifuged. The precipitate was extracted with 4 ml of acetone-water mixture (80:20, v/v) by stirring with a glass rod for 15 min. The sample was again centrifuged, precipitate dissolved in 2 ml of 0.2 M-NaOH and radioactivity and protein content determined. Additional experiments with a mixture of ^{125}I-labelled carrier ceruloplasmin and ^{131}I-labelled albumin demonstrated that with the employment of the TCA-acetone method approximately 75% of albumin was removed while losses of ceruloplasmin did not exceed 10%.

Seromucoid fraction was isolated by the method of Winzler (1955) in the following modification: 2 ml of plasma was mixed with 2 ml of carrier pooled bovine serum (obtained from the animals and stored frozen), diluted with 36 ml of 0.9% NaCl and mixed with 20 ml of 1.8 M-$HClO_4$. The seromucoid was precipitated from the supernatant by adding 12 ml of 5% phosphotungstic acid in 2 M-HCl. The fine sediment was washed with 1% phosphotungstic acid, then with acetone; after drying it was dissolved in 2 ml of 0.2 M-NaOH, and radioactivity and protein were determined.

The total plasma protein was obtained by diluting a 0.5 ml sample to 2 ml with 0.9% NaCl and precipitation with 2 ml of 10% TCA. The precipitate was washed with 5% TCA containing carrier lysine, dissolved in 2 ml of 0.2 M-NaOH, and radioactivity and protein were determined.

Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Measurements of radioactivity. Samples of proteins, 0.1 - 0.2 ml, dissolved in 0.2 M--NaOH were placed in scintillation vials and 10 ml of dioxane-based Bray scintillator fluid was added. ^{3}H radioactivity was determined in a Packard liquid scintillation spectrometer model 2111 with the efficiency of 24%.

Calculations. The counts were computed for albumin, fibrinogen, ceruloplasmin, seromucoid and total proteins in 1 ml of reconstituted blood plasma. Albumin concentration was assumed as 90% of the total protein; this value was estimated from the results of polyacrylamide-gel electrophoresis of the plasma. Due to significant haemolysis during perfusion, the preparations of total proteins, and to a smaller extent of albumin, were contaminated with haemoglobin. The appropriate corrrections were based on the determination of haemoglobin as reduced alkaline haemochromogen (Lemberg & Legge, 1949) in the preparations of total proteins and of albumin.

The final results were expressed as the total activity of [^{3}H]lysine incorporated during 4 h perfusion into a given protein in the whole plasma, and recalculated per 100 g of body weight of the liver donor. To obtain the total volume of the plasma a correction was applied for the amount of plasma trapped in the red cell sediment. The latter value was calculated by multiplying the volume of red cells by the factor 0.28, which was obtained in an experiment with the reconstituted blood containing a known amount of ^{131}I-labelled human albumin.

For statistical analysis, Student's *t*-test was used.

Determination of free [^{3}H]*lysine activity in the liver.* The liver removed from the perfusion apparatus was rinsed with cold saline and quickly homogenized in a Unipan homogenizer for 2 min with 40 ml of cold water. An equal volume of 10% TCA was immediately added, the resulting precipitate spun down and 20 ml of the supernatant was applied to a column (1 × 3 cm) of Dowex 50 WX8 (H^+) prepared according to Kofranyi (1956). Most of amino acids were eluted with 200 ml of 4% pyridine, and then lysine was eluted with 30 ml of 0.62 M-triethylamine. The eluate was evaporated at 37°C and the residue dissolved in 1 ml of water. Descending paper chromatography in phenol saturated with water and carried out in a chamber with NH_3 in air demonstrated the presence of lysine and ornithine, hence lysine could not be directly determined colorimetrically by the Kofranyi (1956) method. It was found, however, that virtually all radioactivity was confined to the lysine spot. Thus the preparation obtained from the Dowex column was used for the estimation of radioactivity, whereas the amounts of lysine and ornithine were determined by column chromatography at pH 4.25 in a Jeol amino acid analyser. The specific activity of lysine was then calculated by dividing counts/ml by μmol/ml of the lysine found, assuming that all the radioactivity was present as free [^{3}H]lysine.

Reagents. Human serum albumin, human γ-globulin, ceruloplasmin (5% solution) and bovine growth hormone (somatotropina) were from Biomed (Warszawa, Poland). The following preparations were from Polfa (Poland): heparin (Warszawa), cortisol (hydrocortisone hemisuccinate, Jelenia Góra), insulin (Tarchomin). Salmine sulphate was from B.D.H. (Poole, Dorset, England), thrombin from Leo (Copenhagen, Denmark). [4,5-^{3}H]L-lysine monohydrochloride (spec. act. 250 mCi/nmol) was obtained from the Radiochemical Centre (Amersham, Bucks., England). ^{125}I--labelled human ceruloplasmin and ^{131}I-labelled human serum albumin were prepared by the ICl method (McFarlane, 1958) using sodium [^{125}I]iodide (14 mCi/μg,

Amersham, Bucks., England) and sodium [^{131}I]iodide carrier free (Świerk, Poland). Bovine serum albumin was from Armour (Eastbourne, England) and amino acids were from Reanal (Budapest, Hungary).

RESULTS AND DISCUSSION

The experiments were carried out in four groups: I and II, on livers from control rats perfused with reconstituted blood, without and with hormones added; III and IV, on livers from turpentine-injected rats perfused with reconstituted blood, without and with hormones, respectively. The results of 5 perfusions for each group are given in Table 1; they are expressed as radioactivity incorporated into particular proteins per 100 g of body weight of liver donor. However, it seemed unjustified to calculate the mean values due to a very large scatter of the results, which possibly reflected individual differences in lysine incorporation. It should be noted that these differences are higher in the present experiments with reconstituted blood than in those of Gordon & Koj (1968) who used full rat blood.

Table 1

The ^{3}H activity in proteins of the reconstituted blood plasma following perfusion of rat liver for 4 h

The results are calculated per 100 g body weight of liver donor. For details see Methods.

Experimental group	Perfusion no.	Protein activity (counts/min/100 g body wt.)				
		Albumin	Fibrinogen	Seromucoid fraction	Cerulo-plasmin	Total protein
I. Control	9	57 400	7 200	13 000	9 300	131 500
	14	72 500	7 180	18 900	12 700	173 000
	15	64 000	4 000	15 200	11 800	138 000
	19	31 300	3 050	6 400	4 600	56 500
	24	43 300	4 890	8 600	6 680	98 000
II. Control+ hormones	10	69 800	6 400	15 600	18 200	124 000
	16	44 200	5 500	8 700	7 100	82 100
	23	29 000	2 790	3 660	3 400	51 000
	26	27 000	1 680	5 080	2 050	54 000
	27	50 000	2 860	7 280	4 330	98 000
III. Turpentine--injected	17	56 100	26 600	30 500	12 300	291 000
	22	49 400	17 800	20 200	9 800	174 000
	29	22 100	6 680	14 400	5 390	116 000
	30	26 500	11 100	8 800	9 700	110 000
	31	26 000	10 900	11 000	5 500	116 000
IV. Turpentine--injected +hormones	18	39 200	22 500	20 400	14 000	193 000
	20	35 000	20 600	21 000	18 100	176 000
	21	37 200	15 900	15 700	14 000	170 000
	25	56 000	31 000	32 600	16 600	282 000
	28	28 400	11 800	15 900	6 100	130 000

When, however, the activities of fibrinogen, seromucoid, ceruloplasmin and total protein were expressed in relative values with respect to the activity of albumin, the scatter within the particular experimental groups became less pronounced as evidenced by the values of standard deviation (Table 2).

It seems that the observed scatter of the results concerning lysine incorporation does not necessarily reflect changes in the absolute rates of protein synthesis but could have been caused by some other factors, e.g. variations in the specific activity of the precursor amino acid inside the liver cell. Hence an attempt was made to isolate free lysine from the liver after perfusion, and to compare its activity with the activity of [^{3}H]albumin in the plasma. However, no direct relationship was found between the specific activity of free[^{3}H]lysine and the activity incorporated into albumin (Table 3). It should be recalled here that Hoffenberg *et al.* (1971), either, found no correlation in the liver between the specific activity of [^{3}H]lysine, the activity incorporated into albumin, and albumin synthesis determined directly by the immunodiffusion method.

Whatever is the reason for the observed high differences in [^{3}H]lysine incorporation within the particular experimental groups, it is clear that the results are more uniform when compared on the relative basis in respect to the radioactivity found in albumin. The advantage of this mode of presentation of data from the experiments *in vivo* has been demonstrated in the measurements of the fibrinogen synthesis rate in rabbits injected with turpentine (Koj, 1968). For accurate evaluation, the lysine content in individual proteins should be taken into consideration; but even without making this correction, the relative values appear to be useful in estimating the degree of acute-phase response.

Table 2

Relative activities of plasma proteins with respect to albumin after 4 h liver perfusion

The data reported in Table 1 were used for calculation of the relative activity for each experiment; mean values ±S.D. are given.

Experimental group	Protein activities relative to albumin			
	Fibrinogen	Seromucoid fraction	Cerulo-plasmin	Total protein
I. Control	0.099 ±0.024	0.225 ±0.025	0.164 ±0.015	2.17 ±0.226
II. Control + hormones	0.086 ±0.027	0.176 ±0.039	0.151 ±0.073	1.87[b] ±0.109
III. Turpentine-injected	0.394[a] ±0.065	0.471[b] ±0.125	0.247[b] ±0.068	4.51[a] ±0.728
IV. Turpentine-injected + hormones	0.511[a, c] ±0.083	0.537[a] ±0.071	0.352[b] ±0.122	4.82[a] ±0.237

[a] $P<0.001$ with respect to the control group.
[b] $P<0.05$ with respect to the control group.
[c] $P<0.05$ with respect to group III.

In agreement with the previously accumulated data (Weimer & Coggshall, 1967; Koj, 1968, 1970b), the subcutaneous injection of turpentine to liver donors evoked a typical acute-phase response, as indicated by a considerable increase in the incorporation of [^{3}H]lysine into fibrinogen and seromucoid, and to a smaller extent into ceruloplasmin. The activity incorporated into albumin was almost unaffected although a decreasing trend could be discerned (see Table 1). A reduced incorporation of labelled amino acids into plasma albumin has been observed in rats subjected to laparatomy (Neuhaus *et al.*, 1966) or injected with talc suspension (Gordon & Koj, 1968).

Stimulation by adrenal steroids of fibrinogen and seromucoid formation in response to turpentine injection was demonstrated in experiments *in vivo* by Weimer & Coggshall (1967). They also drew attention to the importance of the cortisol dose. Jeejeebhoy *et al.* (1972) observed time-dependent differential responses in the synthesis of rat plasma albumin, fibrinogen and transferrin to the administration of cortisol. Jeejeebhoy *et al.* (1970) found also an increased formation of fibrinogen in rats injected with growth hormone. Independently, Atencio *et al.* (1970) reported a dramatic rise in fibrinogen synthesis in rabbits at 5 h after subcutaneous injection of 80 - 100 units of ACTH. According to Wool & Cavicchi (1967), insulin facilitates association of ribosomes and mRNA, and is supposed to stabilize polyribosomes in the perfused liver (van den Borre & Webb, 1972). The enhancement by insulin of the synthesis of plasma proteins during prolonged liver perfusion was demonstrated also by John & Miller (1969).

In our experiments on the effects of hormones (Table 2) it was found that in liver of control rats the mixture of cortisol, insulin and growth hormone had but an insignificant effect on relative lysine incorporation into the investigated plasma

Table 3

Specific activities of free [^{3}H]*lysine in rat livers after 4 h perfusion as compared with* [^{3}H]*albumin activities in the plasma*

Perfusion numbers refer to the data in Table 1. In experiments 26, 27 and 29 duplicate samples from trichloroacetic acid liver extract were analysed. For further details see Methods.

Perfusion no.	Liver extract after Dowex column			Calculated lysine activity (counts/min/μmol)	Plasma albumin ^{3}H activity (counts/min/100 g body wt.)	^{3}H activity ratio plasma albumin / liver lysine
	Lysine (μmol/ml)	Ornithine (μmol/ml)	^{3}H activity (counts/ml)			
26	1.93	0.42	60 200	31 200	27 000	0.865
	1.78	0.34	58 100	32 600		0.828
27	1.75	0.61	28 800	16 500	50 000	3.030
	0.991	0.42	16 000	16 100		3.105
28	1.24	0.40	36 000	29 000	28 400	0.979
29	1.99	1.04	36 800	18 500	22 100	1.194
	1.78	0.77	35 100	19 700		1.122
30	1.45	0.84	27 000	18 600	26 500	1.424
31	0.91	0.74	33 900	37 200	26 000	0.699

proteins. Rather unexpectedly, there was even a decrease in the incorporated activity, but it was statistically significant only in the case of total protein. On the other hand, with the livers derived from turpentine-injected rats, the hormones enhanced the acute-phase response although their effect was statistically significant only for fibrinogen.

The observed slight, statistically insignificant, stimulation by the hormones of [^{3}H]lysine incorporation into ceruloplasmin of turpentine-injected rats was rather unexpected since Evans & Wiederanders (1967) reported an increased content of this protein in plasma of rats following adrenalectomy, and Alias (1971) showed a decrease in plasma ceruloplasmin concentration in rabbits treated with cortisol. However, the situation may be different in the animals challenged with turpentine, where the dose of cortisol, insulin and growth hormone employed in our experiments apparently enhanced the acute-phase protein synthesis in the isolated liver. This fact also indicates that any extrapolation of our results to other experimental conditions may be unjustified, and the experiments with ceruloplasmin synthesis in turpentine-injected rats should be repeated *in vivo*. It should be emphasized that the effect of hormones on protein synthesis is critically dependent on their dose and state of the target tissue.

John & Miller (1969) concluded from their experiments carried out under similar conditions that the acute-phase reactants are synthesized at an increased rate when normal liver is supplied with sufficient amounts of amino acids and the three studied hormones. This supposition is probably too far-fetched since the rise in synthesis of acute-phase reactants started in their experiments only after several hours of perfusion, when the effects of injury inflicted during the operation and isolation procedure began to be manifested. Hormones and amino acids were then required for the expression of potential ability of the already stimulated liver. Hence it seems that the primary factor stimulating the liver cell to produce more acute-phase reactants is still to be identified but, as suggested previously (Gordon & Koj, 1968; Koj, 1970a), it probably originates from the site of injury. The employed hormones exert only a permissive or anabolic action (Weimer & Coggshall, 1967) and represent, in fact, secondary effectors.

The cooperation of Dr. W. Rokicki in preliminary experiments with liver perfusion technique is gratefully acknowledged. The authors are indebted to Dr. W. Klein for carrying out amino acid analysis. Mrs. M. Weigt-Wadas provided technical assistance throughout the study.

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HORMONALNA MODULACJA SYNTEZY BIAŁEK OSTREJ FAZY W PERFUNDOWANEJ WĄTROBIE SZCZURA

Streszczenie

1. Wątroby szczurów normalnych lub nastrzykniętych terpentyną perfundowano przez 4 godziny rekonstytuowaną krwią wzbogaconą w aminokwasy i następnie oznaczano wbudowywanie [^{3}H]lizyny w albuminę osocza, fibrynogen, ceruloplazminę, frakcję seromukoidową oraz białko całkowite.

2. Wstrzyknięcie terpentyny pobudzało syntezę fibrynogenu, seromukoidu i białka całkowitego, podczas gdy radioaktywność albuminy nieznacznie spadała.

3. Dodatek kortyzolu, insuliny i hormonu wzrostowego do rekonstytuowanej krwi nieco zwiększał reakcję ostrej fazy. Wysunięto wniosek, że użyte hormony tylko modulują syntezę glikoproteidów osocza indukowaną odczynem zapalnym, natomiast pierwotne czynniki stymulujące wątrobę pochodzą prawdopodobnie z miejsca uszkodzenia tkanki.

Received 10 December, 1973.

ACTA BIOCHIMICA POLONICA

Vol. 21 1974 No. 2

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REACTION OF NUCLEOSIDES WITH *N*-TRIMETHYLSILYLIMIDAZOLE: SEPARATION OF TMS DERIVATIVES OF ANOMERIC PYRIMIDINE NUCLEOSIDES BY GAS-LIQUID CHROMATOGRAPHY *

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1. The reaction of *N*-trimethylsilylimidazole (TSIM) with various nucleoside analogues was investigated over a temperature range of 20 - 130°C. T. l.c. and g.l.c. analyses showed that the sugar hydroxyls of pyrimidine ribo- and deoxyribonucleosides are quantitatively silylated at room temperature or slightly higher. However, the exocyclic amino group of aminonucleosides and O^4 of 4-ketonucleosides are resistant to TSIM even at 130°C. 2. A general procedure is described for the separation of anomeric pyrimidine *O'*-TMS-nucleosides by g.l.c. with the use of a highly polar column of OV-225, suitable both for analytical and preparative purposes. 3. Di-*O'*-silylated pyrimidine 4-amino-2'-deoxynucleosides are not eluted on g.l.c. columns, due to their low volatility. However, treatment of these nucleosides with TSIM at 120 - 130°C leads to degradation of the pyrimidine ring, with maintenance of the anomeric configuration at C_1 of the pentose ring. These lower molecular weight TMS anomeric products are readily separated on a medium polar OV-17 column, so that this procedure is suitable for analysis of mixtures of anomeric aminodeoxynucleosides. 4. The extension of the foregoing to purine nucleosides, and to analyses of hydrolysis products of nucleic acids, is discussed.

The chemical synthesis of pyrimidine 2'-deoxyribonucleosides, with the aid of suitably protected sugar derivatives and the appropriate pyrimidine, always yields a mixture of the α- and β-anomers of the desired nucleoside, irrespective of the condensation procedure employed. The classical Hilbert-Johnson rearrangement (Hilbert & Johnson, 1930; Prystaš *et al.*, 1965), as well as its modification with the

* This investigation was carried out as Project 09.3.1. of the Polish Academy of Sciences, and profited from the partial support of the Wellcome Trust, the World Health Organization, and the Agricultural Research Service, U.S. Department of Agriculture.

use of silylated pyrimidines (Winkley & Robins, 1968), provides a mixture of the 3′,5′--di-*O*-protected nucleosides with a marked preponderance of the α-anomer. The products obtained by means of the mercury procedure (Fox *et al.*, 1961) usually include a slightly higher proportion of the β-anomer.

The evidence to date points to the presence in natural DNA of only β-2′-deoxynucleosides. It has been reported that irradiation with an unfiltered 254 nm mercury lamp of nucleosides, nucleotides and calf thymus DNA led to the appearance of a very small proportion of α-nucleosides (Sanchez & Orgel, 1970). If the latter were, as appears, the result of some photochemical rearrangement, a most unusual reaction must be involved, and independent confirmation of the foregoing is obviously desirable, in part because of its biological significance. Of more general interest is the demonstration of the existence in yeast RNA of small proportions of the α-anomers of cytidine-2′-phosphate and cytidine-3′-phosphate (Gassen & Witzel, 1965), although nothing is known regarding the possible biological significance of this observation. More recently Seto *et al.* (1972) reported the isolation from culture filtrates of *Streptomyces gryseochromogenes* of 1-(2,3-dideoxy-α-L-glyceropentopyranosyl)cytosine and 1-(3-deoxy-α-L-threopentopyranosyl)cytosine, two cytosine nucleosides with the α-L-configuration.

Separation of nucleoside anomers by paper chromatography is not feasible (Kulikowski & Shugar, 1973). By contrast, ion exchange chromatography has been shown to permit of the fractionation of the anomers of some purine nucleosides (Dekker, 1965; Wolfrom & Winkley, 1967), pointing to the possibility of the development of suitable analytical (and preparative) procedures for α-nucleosides in materials of chemical or biological origin.

Fractionation of anomeric mixtures of unprotected pyrimidine nucleosides by ion exchange chromatography has to date been rather limited. Świerkowski & Shugar (1969) managed to separate the two anomers of 5-ethyldeoxyuridine in crystalline form on a Dowex 1×2 (OH^-) column on a small scale, but subsequent attempts to repeat this were unsuccessful. The method appears to be less capricious with cytosine nucleosides and nucleotides (Sanchez & Orgel, 1970). In any event, it has become almost routine to apply fractionation procedures at the level of the 3′,5′-blocked nucleosides, using bulky protecting groups such as *p*-chlorobenzoyl (Prystaš *et al.*, 1965; Fox *et al.*, 1961), *p*-nitrobenzoyl (Prystaš *et al.*, 1965), *p*-toluyl (Prystaš *et al.*, 1965; Hoffer, 1960), which accentuate the conformational differences between the two anomers. Under these conditions, separations are frequently achieved by fractional crystallization (Hoffer, 1960), but with considerable reductions in yield and at the expense of the biologically more important β-anomer; or by thin-layer chromatography on silica gel (Świerkowski & Shugar, 1969; Kulikowski & Shugar, 1974) or aluminium oxide (Prystaš *et al.*, 1965) which, although usually successful, is extremely tedious and time-consuming. A combination of both the foregoing procedures has also been employed (Kulikowski & Shugar, 1974). It is, of course, clear that such methods, limited as they are to the use of blocked nucleosides, could not readily be applied to analyses of material from biological sources.

Some progress has been achieved in the separation of milligram quantities of unprotected anomeric pyrimidine nucleosides with the aid of t.l.c. on silica gel, using moderately polar solvent systems (Kulikowski & Shugar, 1973).

The foregoing suggested the utility of applying g.l.c. to this problem. Hancock & Coleman (1965) and Sasaki & Hashizume (1966) have reported procedures for full silylation of the sugar and heterocyclic rings in nucleosides, based on trimethylsilylation with a mixture of hexamethylsilazane and trimethylchlorosilane in anhydrous pyridine according to Sweeley *et al.* (1963). The TMS[1] nucleosides obtained in this manner were thermally stable and highly volatile, hence suitable for gas chromatography, but they were extremely sensitive to hydrolysis so that the authors were unable to isolate them with the use of classical techniques. Subsequently Gehrke & Ruyle (1968) utilized *N,O*-bis-(trimethylsilyl)-acetamide (BSA) for derivatization of nucleosides; however, chromatography of thymidine on an SE-30 column gave two non-resolved peaks. Jacobson *et al.* (1968) subsequently applied silylation with BSA, followed by g.l.c., to analysis of the nucleoside composition of enzymic RNA hydrolysates, but encountered difficulties with the stability of silylated cytidine and guanosine.

The application of the foregoing to routine g.l.c. analysis of nucleic acid hydrolysates has been rather limited, because of the convenience of other methods. But the difficulties frequently encountered in the separation of nucleoside anomers pointed to the utility of investigating the possible use of g.l.c. for this purpose.

EXPERIMENTAL

Pyrimidines and pyrimidine deoxynucleosides. Both 1-methyluracil (Ia) and 1-methylcytosine (IIa) were prepared according to the method of Hilbert & Johnson (1930). The syntheses of 5-ethyl-1-methyluracil (Ib) and 5-ethyl-1-methylcytosine (IIb) have been previously reported (Kulikowski & Shugar, 1971). The preparation of 5-ethyl-2′-deoxycytidine (IIIb) and its α-anomer (IIIa), as well as of 5-ethyl-2′-deoxyuridine (IVb) and its α-anomer (IVa), are described elsewhere (Kulikowski & Shugar, 1974). For control purposes the two anomers of 5-ethyl-2′-deoxyuridine, prepared by an alternative route (Świerkowski & Shugar, 1969), were also employed. All compounds were checked for possible trace contaminants by t.l.c (Table 1, see below), and as elsewhere reported (Kulikowski & Shugar, 1971; 1973). The α-anomer of 5-fluoro-2′-deoxyuridine (Va), prepared by Hoffer *et al.* (1959) was a gift from Dr. J.J. Fox. The β-anomer, Vb, was kindly supplied by Hoffman-LaRoche (Nutley, N.J., U.S.A.). Uridine (VIb) was a Reanal (Budapest, Hungary) product, while α-uridine (VIa) was kindly provided by Dr. J. J. Fox. Cytidine (VII) was a Calbiochem (Zürich, Switzerland) product, while thymidine (VIII), 2′-deoxycytidine (IX), adenosine and guanosine were products of Sigma (St. Louis, Mo., U.S.A.).

[1] Abbreviations: TMS, trimethylsilyl; TSIM, *N*-trimethylsilylimidazole; TSIM-Phen, TSIM-phenanthrene; TMCS, trimethylchlorosilane; BSA, *N,O*-bis-(trimethylsilyl)-acetamide.

Thin-layer chromatography. Eastman (Rochester, N.Y., U.S.A.) no. 6060 silica-gel sheets with fluorescent indicator were utilized with a solvent system consisting of analytical grade, redistilled ether-dimethylformamide (8:2, v/v).

Instrumentation. For analytical purposes a Pye-Unicam (Cambridge, England) model 104 gas chromatograph, with a dual hydrogen flame ionization detector, was employed. A model 105 instrument was used for preliminary trials with preparative gas chromatography.

Silylating reagents. These were all products of Pierce Chemical Co. (Rockford, Ill., U.S.A.) and included Silyl-8 g.l.c. column conditioner (no. 38014), and *N*-trimethylsilylimidazole, no. 88623. Phenanthrene, used as control, was a B.D.H. (Poole, England) product and was further recrystallized three times from ethanol and dried over P_2O_5 under vacuum.

Silylation at elevated temperatures was carried out with a Hoeppler ultrathermostat with temp. control to 0.2°C. Aqueous solutions of nucleoside samples (2 mg/200 µl) were first dried in the reaction microvials ("Reacti-Vials" with screw caps and teflon faced discs, total capacity 0.3 ml, cone capacity 0.1 ml, Pierce Chemical Co. no 13220) over P_2O_5 at 760 mm Hg, and then at 10^{-3} mm Hg at room temperature for 18 hours. Samples for gas chromatography were introduced into the columns by means of a Scientific Glass Engineering PTY (Melbourne, Australia) 1 µl syringe fitted with an 11.5 cm needle.

Column materials, packing and conditioning. Column materials were all products of Pierce and consisted of (*A*) 3% OV-225 (no. 49204), coated on 80/100 mesh silanized Chromosorb W (HP) (no. 49095) in borosilicate glass-to-metal columns 1.5 m in length and 4 mm internal diameter; (*B*) 3% OV-17 (no. 49210) on Chromosorb W (HP), column length 1.5 m; (*C*) 10% OV-17 on Chromosorb W (HP), column length 2.7 m; (*D*) 6% SE-30 on Chromosorb W (HP); (*E*) 1% SE-30.

The preparation of 3% (and 10%) OV-17 was as follows: 0.54 g (2 g) OV-17 was completely dissolved in 100 ml acetone in a 250 ml round-bottomed flask. Addition of 18 g Chromosorb W (HP) to the clear solution was followed by careful mixing, and the mixture left for 15 min at room temperature. Solvent was then removed on a water bath at 40 - 45°C, with gentle stirring, at a pressure of 20 mm Hg. The residue was dried for 18 h at 37°C. An analogous procedure was followed for preparation of 3% OV-225 and 6% SE-30 but with acetone and methylene chloride as solvents.

Columns were packed under vacuum (oil pump) with constant vigorous tapping. The opening was then plugged with silanized glass wool, the column inserted into the chromatograph oven, and a flow of argon maintained for 48 h at 250°C. At this temperature, at 1-h intervals, 3 × 10 µl Silyl-8 conditioner was injected and the column conditioned for 18 hours.

Most measurements were made with the OV-17 and OV-225 columns under isothermic conditions. Gas flow rates, unless otherwise indicated, were 40 ml/min argon, 50 ml/min hydrogen, and 600 ml/min air.

Preparation of standard (TSIM-Phen): 5 mg phenanthrene was placed in an 85 × 10 mm test-tube fitted with a collar, a rubber teflon-lined septum and a Nalgene screw-

-cap. The tube was closed, 5 ml TSIM injected with a syringe, and the whole shaken for 5 min to dissolve the phenanthrene.

Silylation of nucleosides: (a) *at room temperature*: 2 mg of the α- or β-anomer of a nucleoside was deposited in a 300-µl "Reacti-Vial", followed by injection of 200 µl TSIM-Phen. The vial was tightly closed and vigorously shaken at room temp. until a clear solution was obtained (15 - 30 min); 1 µl aliquots were withdrawn for g.l.c. (3% OV-17, 185°C) and t.l.c. analyses;

(b) *At elevated temperature*: 2 mg of the α- or β-anomer of a nucleoside was placed in a 300-µl "Reacti-Vial" followed by injection of 100 µl TSIM-phenanthrene. The vial was tightly closed, shaken to dissolve the nucleoside and heated at 120°C or 130°C. At intervals of several hours 1 µl samples were withdrawn and introduced into the chromatograph and the relative molar responses (RMR) calculated. Following completion of the reaction, 1 µl samples were analysed by t.l.c.

Silylation of pyrimidines I(a, b) and II(a, b): 2 mg of 1-methyluracil (Ia) or 5-ethyl-1--methyluracil (Ib) were treated with 200 µl TSIM-Phen at room temperature and/or at 130°C and 1 µl samples withdrawn at various time intervals for chromatography on column *A* at 165°C. The same procedure was followed with 1-methylcytosine (IIa) and 5-ethyl-1-methylcytosine (IIb).

Separation of mixtures of O'-TMS-derivatives of anomeric nucleosides. Method I: Following dissolution of the α-anomer (1.5 mg) at room temp. in 150 µl TSIM-Phen, it was transferred to a solution of the β-anomer (1 mg) in 100 µl TSIM-Phen, the whole well mixed and 1 µl samples injected into column *A* (165°C, argon flow 30 - - 40 ml/min) or column *C* (184°C).

Method II: 2.5 mg of the α-anomer and 1 mg of the β-anomer were combined and shaken with TSIM-Phen at 130°C for 26 h, cooled to room temperature and a 1 µl sample withdrawn for chromatography as above.

RESULTS AND DISCUSSION

Trimethylsilylation of nucleosides has frequently given quantitative yields of the *O*- and *N*-TMS derivatives; but for some nucleosides (usually those with an exocyclic amino group), two peaks were shown by g.l.c. (Hancock & Coleman, 1965). It appeared opportune to attempt development of methods of trimethylsilylation of nucleosides under conditions where the exocyclic amino and/or 4-keto groups are not susceptible to silylation; and to determine the conditions for separation by g.l.c. of anomeric mixtures of the silylated nucleosides.

N-Trimethylsilylimidazole, a very active silylating agent, has hitherto been rarely applied to silylation of hydroxyl groups, and not at all to nucleic acid derivatives. Horning *et al.* (1967) demonstrated that, at 60°C in acetonitrile or pyridine, TSIM silylates only the hydroxyl groups of catecholamines; in contrast to BSA and TMCS, it did not react with primary or secondary amino groups. Furthermore, this reagent does not require the maintenance of strictly anhydrous conditions during the course of the reaction; it has, in fact, been applied to the silylation of aqueous sugar syrups (Brittain & Scheme, 1970). In the present study, it was first essential

to determine whether TSIM would not, at room and elevated temperature, attack the relatively acid ring N_3 hydrogen of 2′-deoxyuridine and uridine, since this would lead to formation of the 3′,5′,O^4-tri-TMS derivative or 2′,3′,5′,O^4-tetra-TMS derivative, the expected lower polarity and higher volatility of which would render more difficult the separation of anomers.

Reaction of TSIM with Ia,b and IIa,b. The foregoing was examined directly by reacting TSIM at 20°C and/or 130°C with 1-methyluracil (Ia) as an analogue of uridine and 5-ethyl-1-methyluracil (Ib) as an analogue of 5-ethyl-2′-deoxyuridine (IVb). Both Ia and Ib dissolved instantaneously in the silylating reagent and, after 15 min reaction at room temperature or 130°C, each was apparently converted quantitatively to a single "new" product with somewhat lower R_F values as shown by t.l.c. (Table 1), and presumably corresponding to the O^4-TMS derivatives of Ia and Ib.

Table 1

T.l.c. of nucleosides and their TMS derivatives, resulting from reaction of nucleosides with N-*trimethylsilylimidazole, on Eastman no. 6060 silica-gel sheets with the solvent system ether - dimethylformamide (8:2, v/v)*

Compound	R_F value	Compound	R_F value
1-Methyluracil (Ia)	0.84	β-Uridine (VIb)	0.32
TSIM solution of Ia	0.66	TMS derivative of VIb	0.76
5-Ethyl-1-methyluracil (Ib)	0.93	β-Cytidine (VII)	0.03
TSIM solution of Ib	0.81	TMS derivative of VII	0.63
1-Methylcytosine (IIa)	0.19	β-Thymidine (VIII)	0.77
5-Ethyl-1-methylcytosine (IIb)	0.25	TMS derivative of VIII	0.85
β-5-Ethyl-2′-deoxycytidine (IIIb)	0.16	β-2′-Deoxycytidine (IX)	0.13
TMS derivative of IIIb	0.68	TMS derivative of IX	0.61
β-5-Ethyl-2′-deoxyuridine (IVb)	0.85	β-Guanosine	0.03
TMS derivative of IVb	0.94	TMS derivative of guanosine	0.69
β-5-Fluoro-2′-deoxyuridine (Vb)	0.78	β-Adenosine	0.31
TMS derivative of Vb	0.84	TMS derivative of adenosine	0.76

When these reaction products were subjected to g.l.c. on column *C* at 184°C, each gave a single peak (Table 2). However, both Ia and Ib themselves proved sufficiently volatile to elute from such a column and, to our surprise, exhibited R_T values virtually identical with those obtained following treatment with TSIM (Fig. 1, Table 2). Furthermore, when the products of reaction of Ia and Ib with TSIM were eluted from the t.l.c. chromatograms with anhydrous chloroform and their u.v. spectra examined, their λ_{max} values differed from those for untreated Ia and Ib by only 1 nm. If the O^4-silylated derivatives had been formed by treatment with TSIM, one would have anticipated a difference of the order of 7 nm, which is the difference in λ_{max} values between 1-methyluracil and 1-methyl-2-keto-4-ethoxypyrimidine (Shugar & Fox, 1952).

Table 2

G.l.c. of TMS derivatives of anomeric pyrimidine ribo- and deoxyribonucleosides

The nucleosides were silylated with *N*-trimethylsilylimidazole (TSIM) at 20°C and/or 130°C. Further details in text. R_T, retention times; R_{r_T}, relative retention times with respect to phenanthrene.

Compound	Silylation temp. (°C)	Column composition							
		10% OV-17		3% OV-225		6% SE-30		1% SE-30	
		R_T	R_{r_T}	R_T	R_{r_T}	R_T	R_{r_T}	R_T	R_{r_T}
α-5-Ethyl-2′-deoxycytidine (IIIa)	130	47′	1.078			10′05″[a]	1.870		
β-5-Ethyl-2′-deoxycytidine (IIIb)		56′01″	1.285						
Phenanthrene		43′36″	1.000				1.000		
α-5-Ethyl-2′-deoxyuridine (IVa)	130	49′49″	1.142			10′05″[a]	1.870		
β-5-Ethyl-2′-deoxyuridine (IVb)		56′36″	1.298						
Phenanthrene		42′33″	1.000			5′23″	1.000		
α-5-Fluoro-2′-deoxyuridine (Va)	130	50′10″	1.132			9′37″[a]	1.783		
β-5-Fluoro-2′-deoxyuridine (Vb)		57′47″	1.304						
Phenanthrene		44′18″	1.000			5′23″	1.000		
1-Methyluracil (Ia)	20 - 130	18′24″	0.472						
5-Ethyl-1-methyluracil (Ib)		20′30″	0.526						
Phenanthrene		39′	1.000			5′23″	1.000		
α-Uridine (VIa)	20			4h57′15″	19.050				
β-Uridine (VIb)				5h27′	20.960			2′11″	1.135
β-Cytidine (VII)								11′10″	5.818
Phenanthrene				15′36″	1.000			1′55″	
α-5-Ethyl-2′-deoxyuridine (IVa)	20			6h 8′50″	19.790				
β-5-Ethyl-2′-deoxyuridine (IVb)				6h37′	21.600				
Phenanthrene				15′36″	1.000				
α-5-Fluoro-2′-deoxyuridine (Va)	20			4h52′45″	18.770				
β-5-Fluoro-2′-deoxyuridine (Vb)				5h19′40″	20.490				
Phenanthrene				15′36″	1.000				

[a] The two anomers were not resolved on this column.

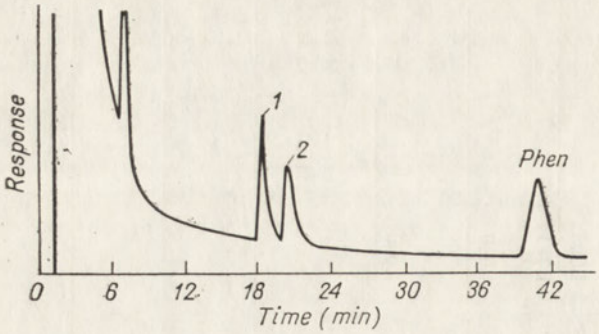


Fig. 1. G.l.c. chromatogram of 1-methyluracil (Ia; peak *1*) and 5-ethyl-1--methyluracil (Ib; peak *2*), on a 2.7 m column of 10% OV-17 at an isotherm of 185°C; argon flow 40 ml/min, attenuation 10^{-9} a.f.s., with phenanthrene (Phen) as internal standard. *Note*: when either Ia or Ib was first treated with TSIM at 20 - 130°C, each gave the same peak as for the untreated compound.

In addition, if Ia and Ib were converted by TSIM to the corresponding O^4-silylated derivatives, their R_F values on t.l.c. should have increased, and not decreased as observed (Table 1). This led to the suspicion that the decreased R_F values on t.l.c. might be due to some type of non-covalent interaction between Ia (or Ib) and TSIM or the product of its degradation, i.e. imidazole. To check this, 1,3-dimethyluracil was reacted with TSIM and then subjected to t.l.c. with 95 : 5 ether-dimethylformamide; under these conditions the dimethyluracil spotted from a methanolic solution migrated with the solvent front, while the same compound treated with TSIM exhibited an R_F of about 0.60. It may, consequently, be concluded that neither Ia nor Ib undergo silylation on treatment with TSIM either at room temperature or 130°C. Reaction of 5-ethyl-1-methylcytosine (IIb), the analogue of 5-ethyl-2′--deoxycytidine (IIIb), with TSIM was equally without effect, even when the reaction time was extended to 48 h. Since it might be argued that the 5-ethyl substituent sterically hinders silylation of the neighbouring exocyclic amino group, 1-methylcytosine (IIa) was treated with TSIM under identical conditions; again only the starting compound was recovered intact.

Reaction of nucleosides with TSIM.

(a) *Silylation of ribonucleosides*: Uridine (VIb) and its α-anomer (VIa) were each taken up in 175 mole excess of TSIM (without solvent) and shaken vigorously at room temperature until completely dissolved (about 15 min), following which t.l.c. demonstrated the complete disappearance of both starting compounds and the formation from each of a new product, both of which exhibited tailing. When subjected to g.l.c. on column *E* at 215°C, each product gave a single peak (Table 2). Heating of the products with TSIM at 120°C for 2 h did not alter either the relative retention times (R_{r_T}) or the relative molar response (RMR). On the other hand, silylation of cytidine (VII) and aminopurine ribonucleosides was only partial at room temperature; full silylation in these cases required a reaction temperature of 60°C or higher and a longer reaction time (2 h). It is worth noting that the products of silylation of uridine (VIb) and cytidine (VII) were very well resolved on column *E* at 215 - 235°C (Fig. 2); the appreciably lower value of R_{r_T} for uridine, as well as the sharpness of its peak relative to that for cytidine (Fig. 2) is consistent with the higher degree of volatility of the former.

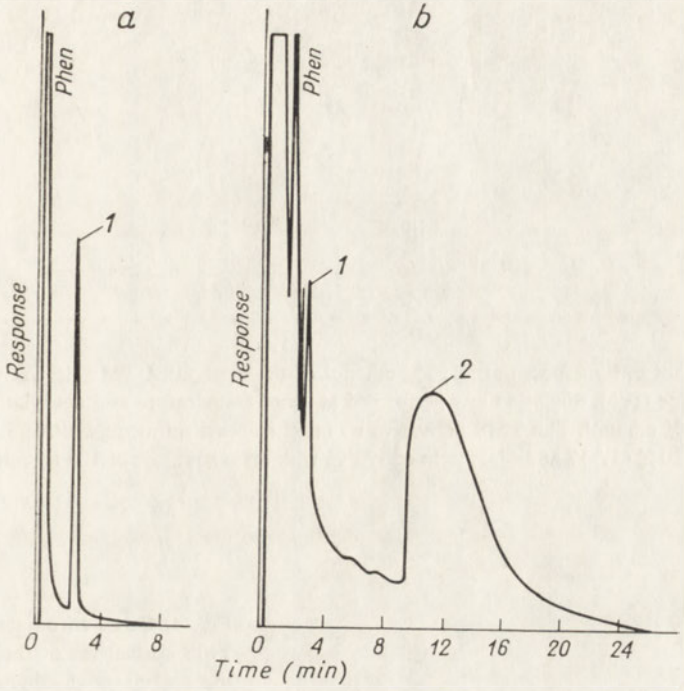


Fig. 2. G.l.c. chromatograms of the *O'*-TMS derivatives of: (*a*) uridine (peak *1*), and (*b*) cytidine (peak *2*) with phenanthrene (Phen) as internal standard, on a 1.5 m column of 1% SE-30 at an isotherm of 214°C, with argon flow of 40 ml/min and attenuation 10^{-9} a.f.s. *Note*: the cytidine sample used contained a trace contamination of uridine.

(b) *Silylation of 2'-deoxynucleosides*: (i) *at room temperature*: The nucleosides III, IV and V, as well as the corresponding α-anomers, were each shaken with a 175 molar excess of TSIM to obtain clear solutions (15 - 30 min), following which t.l.c. (Table 1) demonstrated quantitative conversion of each of the nucleosides to a single new product. The products of silylation of the ketonucleosides IVa,b, Va,b and of thymidine (VIII) on column *C* at 184°C gave peaks with relatively long retention times, of the order of 1.5 - 1.8 relative to phenanthrene (Table 2). By contrast, the products of silylation of the aminodeoxynucleosides IIIa,b and 2'-deoxycytidine (IX) were identified only by t.l.c. (Table 1), since they could not be detected by g.l.c. under any of the conditions employed, due undoubtedly to the fact that each of these contains only two trimethylsilyl groups, with consequent low volatility. The products of the room temperature reaction of IVa and IVb with TSIM were separated by preparative g.l.c. on a 20% OV-17 column at 190°C with an argon flow of 120 ml/ /min, and desilylated by heating for 1 h in aqueous ethanol. The u.v. spectra of both the desilylated products in 95% ethanol were found to be identical with the u.v. spectrum of 5-ethyl-2'-deoxyuridine (Fig. 3).

(ii) *At elevated temperatures*: Heating of the TSIM solutions of the ketonucleosides IVa,b, Va,b, and VIII at 120 - 130°C led to the gradual disappearance of the g.l.c. peaks corresponding to the products of silylation at room temperature

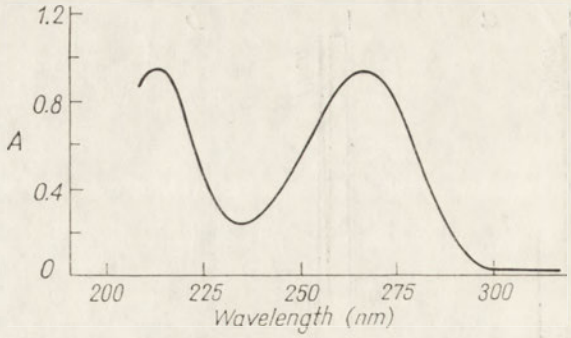


Fig. 3. U.v. absorption spectrum in 95% ethanol of the desilylated TMS derivative of β-5-ethyl-2′-deoxyuridine (IVb). Silylation was performed at room temperature and desilylation by refluxing for 1 h in 95% ethanol. The TMS derivative was first isolated on a preparative 3.5 m column of 20% OV-17 at an isotherm of 190°C, with an argon flow of 120 ml/min.

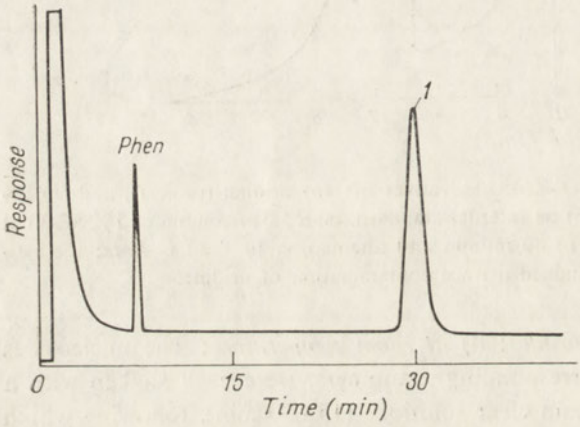


Fig. 4. G.l.c. chromatogram of the TMS derivatives of the α- and β-anomers (3:5) of 5-ethyl-2′-deoxyuridine (IVa,b, peak *1*) with phenanthrene (Phen) as internal standard. Silylation was performed at room temperature. For chromatography, a 2.7 m column of 10% OV-17 was used, at an isotherm of 242°C, with an argon flow of 40 ml/min, attenuation 2×10^{-9} a.f.s. *Note*: the two anomers are not resolved on this column.

(Fig. 4), and the concomitant appearance for each of a new peak with a considerably lower retention time (Fig. 5, peak *1*). When the aminonucleosides IIIa,b and IX (the silylation products of which, as pointed out in the previous section, are not detectable by g.l.c.) were treated with TSIM at 120 - 130°C, they now gave g.l.c. peaks with low retention times (Fig. 6a), the RMR values of which increased with time of heating (Fig. 6b).

It should be emphasized that the products of silylation with TSIM at 120 - 130°C of the 2′-deoxynucleosides with a 5-ethyl pyrimidine substituent, i.e. IIIa,b and IVa,b, exhibited R_{r_T} values identical for the two α-anomers, and similarly for the two β-anomers; whereas for the anomeric pair with a 5-fluoro substituent (i.e. Va,b) the R_{r_T} values for the α- and β-anomers were slightly lower (Table 2).

The products of reaction of IIIa and IIIb with TSIM at 120 - 130°C (Fig. 7, peaks *1* and *2*) were isolated by preparative g.l.c., desilylated by heating in aqueous ethanol, and the u.v. spectra of the desilylated products recorded in 95% ethanol. The spectra,

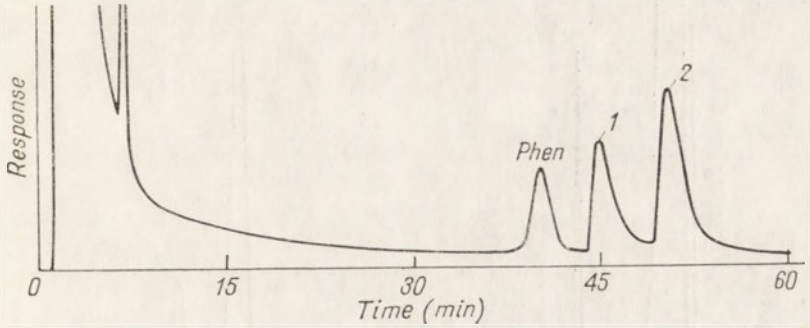


Fig. 5. G.l.c. chromatogram of the TMS derivatives of the α- and β-anomers (2:3) of 5-ethyl-2′-deoxyuridine (IVa and IVb, peaks *1* and *2*), referred to in text as X-TMS derivatives, with phenanthrene (Phen) as internal standard. Silylation was performed at 130°C. For chromatography, a 2.7 m column of 10% OV-17 was used at 185°C; argon flow 40 ml/min; attenuation 5×10^{-10} a.f.s. Each anomer was first reacted separately with TSIM until it gave a constant RMR value *vs* Phen. The two TSIM-reacted solutions were then mixed and 1 μl injected into the column.

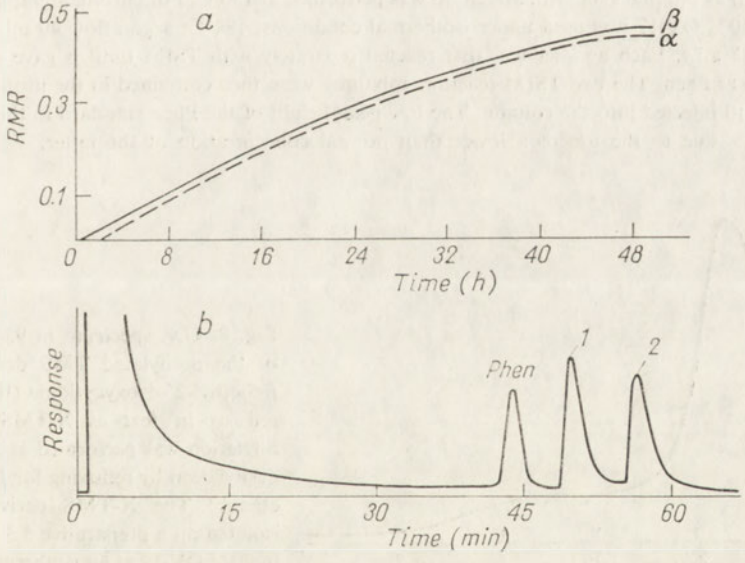


Fig. 6. Kinetics of formation (*a*), and g.l.c. chromatogram (*b*) of the TMS derivatives of the α- and β-anomers of 5-ethyl-2′-deoxycytidine (IIIa and IIIb), referred to as X-TMS derivatives. Silylation was performed at 120°C. (*a*), Each anomer was treated separately with TSIM and formation of the corresponding X-TMS derivative followed with time by g.l.c. (*b*), The solutions of α- and β-anomer were combined in the proportion IIIa:IIIb=3:2, following maximal formation of the X-TMS derivatives as determined from the kinetic curves in (*a*); 1 μl was injected into a 2.7 m column of 10% OV-17 at an isotherm of 183°C, argon flow 40 ml/min, attenuation 10^{-9} a.f.s. Peak *1*, α-anomer; peak *2*, β-anomer. Phen, phenanthrene added as an internal standard.

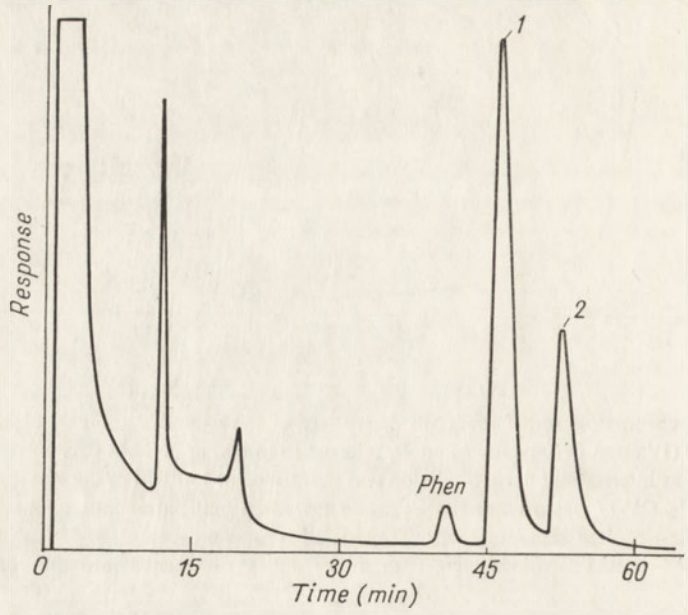


Fig. 7. G.l.c. chromatogram of the TMS derivatives of the α- and β-anomers (5:2) of 5-ethyl-2′--deoxycytidine (IIIa, peak *1*; IIIb, peak 2), referred to in text as X-TMS derivatives, with phenanthrene (Phen) as internal standard. Silylation was performed at 130°C. For chromatography, a 2.7 m column of 10% OV-17 was used under isothermal conditions, 184°C; argon flow 40 ml/min; attenuation 10^{-9} a.f.s. Each anomer was first reacted separately with TSIM until it gave a constant RMR value *vs* Phen. The two TSIM reaction mixtures were then combined in the proper proportions and 1 μl injected into the column. The low peak height of the Phen standard in this case was due to the use of a lower than normal concentration of the latter.

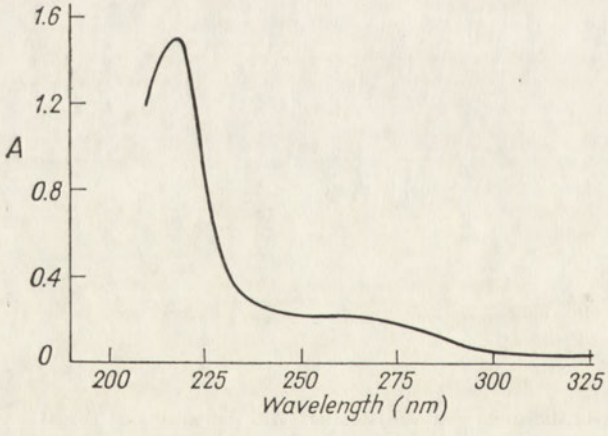
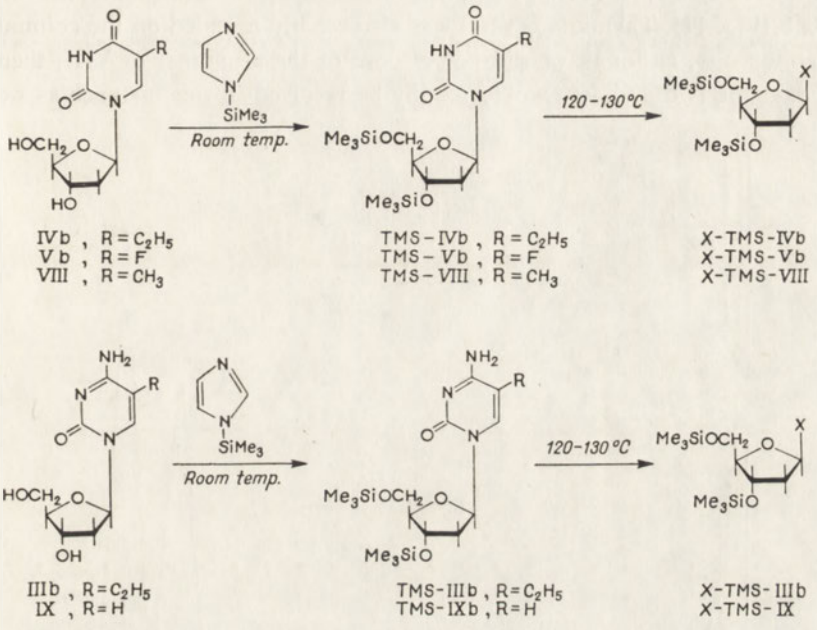


Fig. 8. U.v. spectrum in 95% ethanol of the desilylated TMS derivative of β-5-ethyl-2′-deoxycytidine (IIIb), referred to in text as X-TMS product. Silylation was performed at 130°C and desilylation by refluxing for 1 h in 95% ethanol. The X-TMS derivative was isolated on a preparative 3.3 m column of 20% OV-17 at an isotherm of 190°C, with an argon flow of 120 ml/min.

virtually identical for both anomeric products (Fig. 8) show only end absorption in the quartz ultraviolet, with a maximum at 218 nm, indicative of saturation of the 5,6 bond, or of degradation, of the pyrimidine rings.

The fact that the products of reaction of the two anomers at elevated temperatures give retention times appreciably lower than the products silylated at lower tempera-

tures, pointing to lower molecular weights of the former, suggests degradation of the pyrimidine ring at 120 - 130°C, with elimination of some fragment at C_4 and formation of X-TMS derivatives in which the original anomeric configurations at C_1 of the pentose ring are maintained (Scheme 1). Such a mechanism is consistent with the observation that the relative retention times of the α- and β-anomers of the X-TMS products correspond to those of the intact anomeric nucleosides silylated at lower temperatures without degradation.



Scheme 1. Proposed scheme for reaction of a pyrimidine deoxyribonucleoside with TSIM at 120 - - 130°C, leading to degradation of the pyrimidine ring and formation of an X-TMS derivative with the same anomeric configuration as the original nucleoside. The reaction in this case is for a β-anomer, and would be similar for the α-anomer.

The foregoing behaviour was exhibited not only by the anomeric aminodeoxynucleosides IIIa,b, but also by all the ketodeoxynucleosides. The possibility that degradation of the aminodeoxynucleosides proceeds *via* initial deamination is excluded by the fact that, during degradation, no products are observed with retention times corresponding to those for *O'*-TMS derivatives of the corresponding ketodeoxynucleosides IVa,b.

Although the structure of X (Scheme 1) has not as yet been elucidated, the foregoing may be profited from for analytical purposes with anomeric aminodeoxynucleosides, as described below.

G.l.c. separation of anomeric nucleosides silylated with TSIM at room temperature. Separation of the *O'*-TMS-derivatives of anomeric pyrimidine nucleosides by g.l.c. required the application of special procedures. Use of conditions normally applied to separation of nucleic acid derivatives by g.l.c. (Hancock & Coleman, 1965; Sasaki

& Hashizume, 1966; Gehrke & Ruyle, 1968; Horning *et al.*, 1967) were quite ineffective for these pairs of anomers either under isothermic conditions or by programming. The usual 1.5 m columns of 1% SE-30, 3 - 10% OV-17 or OV-210, in the temperature range 150 - 235°C, did not give any separation of the mixture of two anomeric derivatives. Satisfactory separations were obtained only with the use of a 1.5 m column of the strongly polar 3% OV-225. Under these conditions satisfactory separations were achieved for the anomeric pairs of the silylated keto-ribonucleosides VIa and VIb (Fig. 9) and keto-2′-deoxyribonucleosides IVa,b (Fig. 10) and Va,b (Fig. 11). Thymidine (VIII) was also readily revealed on the column under these conditions; although we did not dispose of the α-anomer of VIII, there is no doubt that the two anomers would readily be resolved in this instance as well.

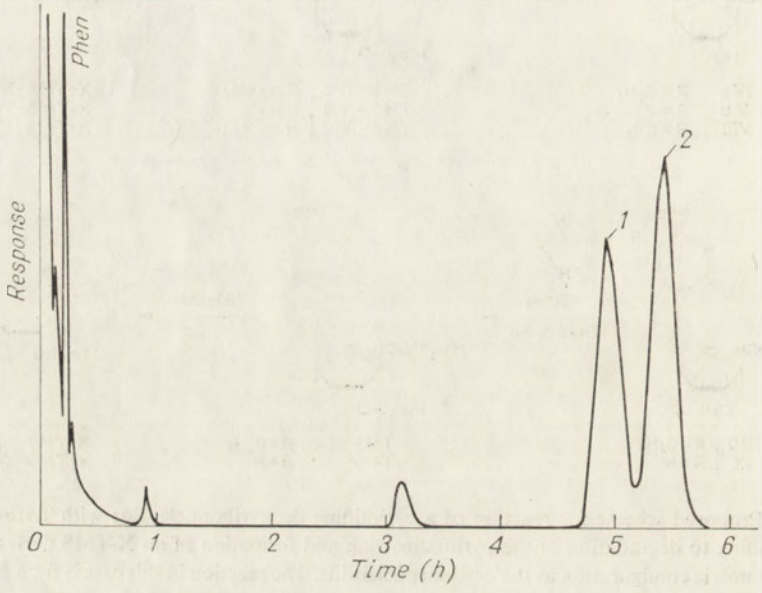


Fig. 9. G.l.c. chromatogram of the TMS derivatives of the α- and β-anomers (2:3) of uridine (VIa, peak *1* and VIb, peak *2*) with phenanthrene (Phen) as internal standard. Silylation was performed at room temperature. For chromatography, a 1 µl sample was injected into a 1.5 m column of 3% OV-225 at an isotherm of 165°C; argon flow 40 ml/min, attenuation 10^{-9} a.f.s.

G.l.c. separation of anomeric deoxynucleosides silylated with TSIM at 120 - 130°C (X-TMS derivatives). As mentioned above, the *O′*-TMS derivatives of the aminodeoxynucleosides IIIa,b and IX could not be eluted from the g.l.c. columns, probably as a result of their low volatility. However, prolonged heating of these nucleosides with TSIM at 120 - 130°C led to decomposition products with shorter retention times, relative to phenanthrene (Figs. 6 and 7, peaks *1* and *2*), than the intact nucleosides silylated at room temperature. This observation is of considerable interest and obviously calls for further study to establish the nature of the decomposition product(s) X (see Scheme 1). What is of particular significance is the fact

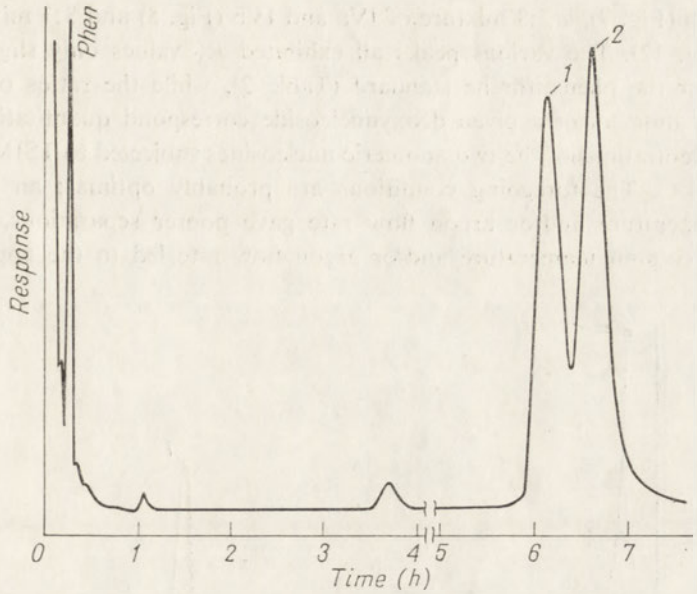


Fig. 10. G.l.c. chromatogram of the TMS derivatives of the α- and β-anomers (2:3) of 5-ethyl-2′--deoxyuridine (IVa, peak *1* and IVb, peak *2*) with phenanthrene (Phen) as internal standard. Silylation was performed at room temperature. For chromatography, a 1 μl sample was injected into a 1.5 m column of 3% OV-225 at an isotherm of 165°C; argon flow 40 ml/min, attenuation 10^{-9} a.f.s.

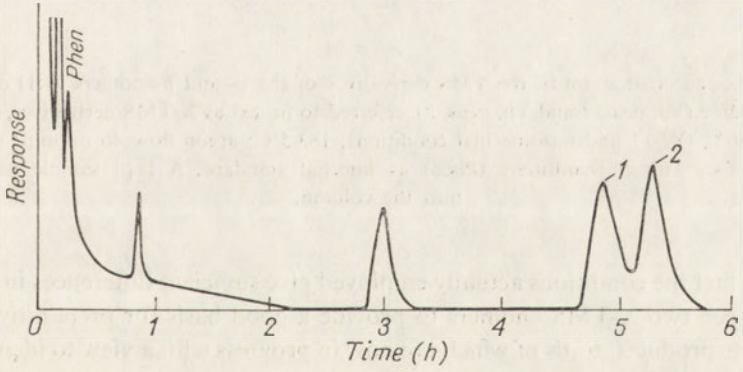


Fig. 11. G.l.c. chromatogram of the TMS derivatives of the α- and β-anomers (2:3) of 5-fluoro-2′--deoxyuridine (Va, peak *1* and Vb, peak *2*), with phenanthrene (Phen) as internal standard. Silylation was performed at room temperature. For chromatography, a 1 μl sample was injected into a 1.5 m column of 3% OV-225 at an isotherm of 165°C; argon flow 40 ml/min, attenuation 10^{-9} a.f.s. *Note*: the high background at R_T of about 50′ originates from the silylating reagent; the origin of the background at an R_T of 3 h is not clear.

that the X-TMS derivatives of the α- and β-anomers of a given deoxynucleoside can be separated on a 2.7 m column of the polar 10% OV-17 with an argon flow of 40 ml/min under isothermal conditions at 183 - 185°C. Trial runs under these conditions gave good separations of the X-TMS derivatives of a 5:2 mixture of

IIIa and IIIb (Fig. 7), a 2:3 mixture of IVa and IVb (Fig. 5) and 3:1 mixture of Va and Vb (Fig. 12). The various peaks all exhibited R_T values only slightly higher than that for the phenanthrene standard (Table 2), while the ratios of the areas for the two anomers of a given deoxynucleoside correspond quantitatively to the relative concentrations of the two anomeric nucleosides subjected to TSIM treatment at 120° - 130°C. The foregoing conditions are probably optimal; an increase in column temperature and/or argon flow rate gave poorer separations, whereas a decrease in column temperature and/or argon flow rate led to the appearance of

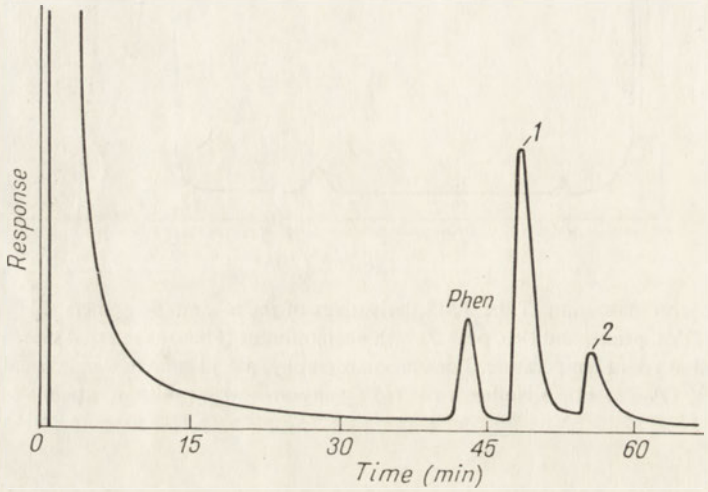


Fig. 12. G.l.c. chromatogram of the TMS derivatives of the α- and β-anomers (3:1) of 5-fluoro--2′-deoxyuridine (Va, peak *1* and Vb, peak *2*), referred to in text as X-TMS derivatives, on a 2.7 m column of 10% OV-17 under isothermal conditions, 183.5°C; argon flow 40 ml/min; attenuation 5×10^{-10} a.f.s., with phenanthrene (Phen) as internal standard. A 1 μl sample was injected into the column.

tailing. In fact the conditions actually employed give sufficient differences in retention times between two X-TMS anomers to provide a good basis for preparative separation of these products, trials of which are now in progress with a view to identification of X. Meanwhile the foregoing procedure is at the moment the only one which can be applied to the g.l.c. analytical determination of the anomeric ratio of pyrimidine aminodeoxynucleosides.

The present investigation demonstrates that trimethylsilylation with TSIM of 4-amino and 4-keto nucleosides is a selective process leading to substitution only of the sugar hydroxyls, without affecting the O^4 of the pyrimidine ring or the exocyclic amino group. Formation of the *O′*-TMS derivatives of the pyrimidine nucleosides at temperatures not much above room temperature is essentially quantitative, a finding of some importance in relation to thermally labile nucleosides. Preliminary trials demonstrated the need for higher temperatures with purine nucleosides, about 60°C or somewhat higher.

When to the foregoing is added the fact that the use of this silylating reagent obviates the need for strictly anhydrous conditions, it appears to be one of the best (if, in fact, not the best) reagents for silylation of the sugar hydroxyls of nucleosides. It remains to establish whether its specificity extends equally to purine nucleosides; preliminary experiments along these lines are sufficiently promising to warrant further studies. It should, however, be emphasized that the known ability of a strongly basic anion exchanger to separate anomeric purine nucleosides (Dekker, 1965) raises some doubts as to the utility of g.l.c. for this purpose.

The use of a 3% OV-225 column at 160 - 170°C provides satisfactory separations of the *O'*-silylated anomeric pyrimidine ribo- and 2'-deoxyribonucleosides. The long retention times required for such separations with the 4-keto nucleosides are unavoidable, but this disadvantage is clearly outweighed by the good separations achieved, and the facility with which the procedure may be applied. It is possible that the retention times may be reduced by the use of another silylating reagent which derivatizes also the 4-keto groups.

The inability to achieve good separations with intact pyrimidine aminodeoxynucleosides is, as pointed out above, due simply to the fact that such derivatized nucleosides contain only two silyl groups and are, consequently, not sufficiently volatile. In these instances one may apply TSIM treatment at elevated temperatures (120 - 130°C) to obtain quantitatively lower molecular weight silylated products, with maintenance of the anomeric configuration, which are readily resolved into the two anomers. Naturally this procedure is limited only to analytical applications. It should be noted also that retention times for these X-TMS products are considerably lower than for the intact derivatized nucleosides (Table 2). This could be due not only to the lower molecular weights of the X-TMS derivatives (Scheme 1), but also possibly to the possession by these derivatives of an additional silyl group on the X-moiety. It is of interest in this connection that Harvey & Horning (1973) have recently demonstrated the separation, on a 1% OV-17 column, of anomeric pairs of TMS derivatives of some sugar phosphates.

We are indebted to Dr. J. J. Fox for gifts of α-uridine and α-5-fluoro-2'-deoxyuridine, and to Hoffman-LaRoche (Nutley, N. J.) for a sample of β-5-fluoro-2'-deoxyuridine. We should also like to acknowledge the excellent technical assistance of Mrs. Maria Żylonis and Miss Magdalena Piwnicka.

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REAKCJA NUKLEOZYDÓW z *N*-TRÓJMETYLOSYLILOIMIDAZOLEM: ROZDZIAŁ TMS POCHODNYCH ANOMERYCZNYCH NUKLEOZYDÓW PIRYMIDYNOWYCH METODĄ CHROMATOGRAFII GAZ - CIECZ

Streszczenie

1. Zbadano działanie trójmetylosyliloimidazolu (TSIM) na nukleozydy — składniki i analogi kwasów nukleinowych w zakresie temperatur 20 - 130°C. Wykazano przy pomocy analizy t.l.c. i g.l.c., że u wszystkich badanych rybo- i dezoksyrybonukleozydów pirymidynowych wyłącznie grupy hydroksylowe cukrów reagują z TSIM w temperaturze pokojowej lub nieco wyższej. Egzocykliczny atom tlenu O^4 oraz grupa aminowa w pozycji 4 tych nukleozydów nie jest podstawiana przez TSIM nawet w temp. 130°C.

2. Opracowano nową, uniwersalną metodę rozdziału anomerycznych *O'*-TMS nukleozydów opartą na g.l.c. przy użyciu silnie polarnej kolumny OV-225. Dla anomerycznych dwu-*O'*-TMS-4--amino-2'-dezoksynukleozydów, które nie są eluowane z kolumn g.l.c. nawet w drastycznych warunkach, opracowano metodę rozdziału opartą na ilościowej degradacji w 120 - 130°C do niżej cząsteczkowych TMS pochodnych o zachowanej wyjściowej konfiguracji przy C_1, których rozdział uzyskano na średnio polarnej kolumnie OV-17. Ta ostatnia procedura może być stosowana do analizy mieszaniny anomerycznych aminodezoksynukleozydów.

3. Opisano możliwości zastosowania tych metod do analizy hydrolizatów i produktów degradacji kwasów nukleinowych.

Received 14 December, 1973.

ACTA BIOCHIMICA POLONICA

Vol. 21 1974 No. 2

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PROPERTIES OF DIASTEREOISOMERIC PHOTOHYDRATES OF URACIL NUCLEOSIDES *

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The two diastereoisomeric photohydrates of 2′-deoxyuridine were isolated by thin-layer chromatography and both obtained in crystalline form. The kinetics of thermal dehydration of both isomers in neutral and acid media were studied. It was found that, in acid medium, isomer II does not undergo direct dehydration, but is transformed to isomer I, which then undergoes acid-catalysed dehydration. Evidence is presented, in agreement with previous observations, that this conversion of isomer II to isomer I proceeds *via* opening of the N_1-C_6 bond of the uracil ring.

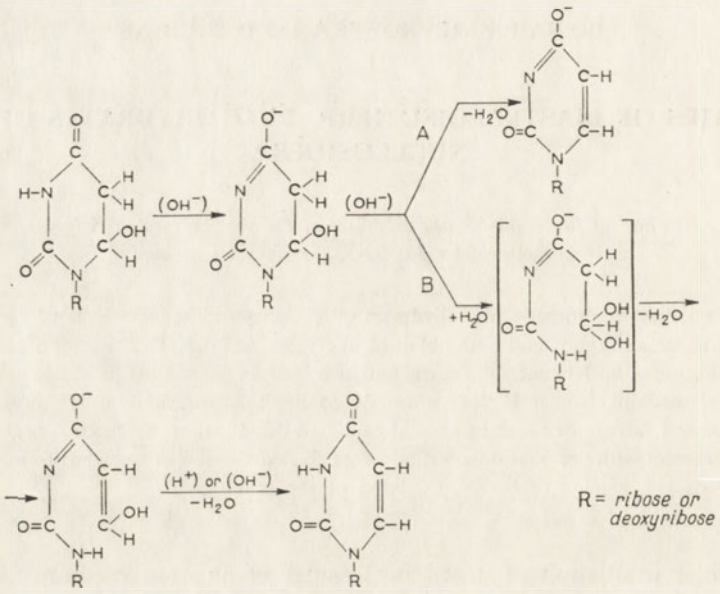
Ultraviolet irradiation of uracil nucleosides in aqueous medium leads to the formation of the photohydrate, 5,6-dihydro-6-hydroxyuridine (Shugar, 1960). The water adduct may subsequently be eliminated in the dark, in neutral or acid medium, by what is essentially an acid-base catalysed reaction (Wierzchowski & Shugar, 1961; Wechter & Smith, 1968) to regenerate the parent nucleoside. In alkaline medium the dark elimination reaction is more complex (Fikus & Shugar, 1966). In the presence of monovalent Na^+ or K^+ cations, the alkali-catalysed dehydration is not quantitative, a fact utilized by Schuster (1964) to estimate the number of uracil hydrates formed in u.v.-irradiated polynucleotides. In the presence of NH_4^+, on the other hand, alkali-catalysed dehydration results in quantitative regeneration of the parent nucleoside *via* two different pathways (Fikus & Shugar, 1966): (a) by direct elimination of the water molecule from the 5,6 bond, and (b) *via* an intermediate with an absorption band at 290 nm ($interm_{290}$)[1]. The structure of this intermediate,

* This work was supported by the Polish Academy of Sciences within the project 09.3.1 and profited from the partial support of the World Health Organization, The Wellcome Trust, and the Agricultural Research Service, U. S. Department of Agriculture.

[1] The following abbreviations are used: UdR, 2′-deoxyuridine; UR, uridine; UdR·H_2O, 5,6-dihydro-6-hydroxydeoxyuridine, the photohydrate of UdR, and similarly for UR·H_2O; UdR·H_2, 5,6-dihydro-2′-deoxyuridine; PDAB, *p*-dimethylaminobenzaldehyde; $interm_{290}$, the product of ring opening of UdR·H_2O or UR·H_2O in alkaline medium, with an absorption maximum at 290 nm (Scheme 1).

which is formed by opening of the N_1-C_6 bond of the hydrated uracil ring, has been established. The open-chain intermediate is unstable and spontaneously dehydrates, following which it undergoes ring closure to regenerate the parent nucleoside. The entire reaction scheme (Fikus & Shugar, 1966) is shown in Scheme 1.

In the foregoing, no consideration was given to the possibility of formation of isomeric photohydrates. The occurrence of this isomerism was demonstrated by Chambers (1968) and confirmed by Wechter & Smith (1968).



Scheme 1

It was subsequently found that the photohydrates of various uracil nucleosides could be separated by t.l.c. into two products, which were the presumed diastereoisomers of the photohydrates. The two isomers differed in stability, while each underwent alkali-catalysed elimination of the water molecule by one of the two pathways shown in Scheme 1. Furthermore, one of the isomers of the photohydrate of deoxyuridine (UdR) was isolated in crystalline form and some of its properties described (Pietrzykowska & Shugar, 1969). In particular it was shown that this isomer, denoted as isomer I, underwent alkaline dehydration *via* pathway B in Scheme 1; whereas the second isomer, denoted as II, dehydrated in alkaline medium *via* path A.

The present communication describes the isolation in crystalline form of the second isomeric photohydrate of UdR and presents some additional properties of both isomeric photohydrates.

MATERIALS AND METHODS

Deoxyuridine was obtained from Waldhof Zellstoffabrik (Mannheim, G.F.R.) and D_2O, with a mole % content of ^2H in excess of 99.7%, from Koch-Light (Colnbrook, England).

5,6-Dihydrodeoxyuridine was prepared by hydrogenation of a 10^{-3} M solution of UdR in the presence of rhodium on charcoal, as described by Cohn & Doherty (1956).

Ultraviolet absorption spectra were obtained with the aid of a Unicam SP-500 instrument fitted with a specially constructed cuvette compartment, through which was circulated an aqueous glycol mixture fed from a Hoeppler ultrathermostat. A calibrated thermistor in a dummy cuvette was used to measure the temperature in the reaction cuvette.

O.r.d. spectra were recorded on a JASCO ORD/UV-5 instrument, using 2.5×10^{-4} M aqueous solutions at 10°C.

Kinetics of dehydration of 10^{-4} M solutions of the UdR photohydrates were measured spectrally with the Unicam SP-500, with the cuvette compartment at 80°C, and monitoring the appearance of the 262 nm band of UdR. During the period required to attain the temperature of 80°C, the increase in A_{262} varied from 2 - 4% of that required to attain maximal reversal after about 3 hours. For dehydration kinetics in acid medium (pH 1, 0.1 M-HCl), the H_2O solutions were acidified with 0.01 volume of conc. HCl; whereas the D_2O solutions were acidified with 0.015 volume of HCl, to take account of the increased basicity of D_2O (Wiberg, 1955), so that the pH or pD of the two solutions were the same.

Colour reactions of the various compounds with *p*-dimethylaminobenzaldehyde were carried out as described by Fink *et al.* (1956). About 30 µg of substance was deposited on each of two strips of Whatman paper no. 1. One strip was first sprayed with a solution of 0.5 M-NaOH and dried. Both strips were then sprayed with the *p*-dimethylaminobenzaldehyde (PDAB) reagent.

RESULTS

Isolation of UdR photohydrates. The procedure for isolation of the UdR photohydrate, and the separation of the diastereoisomers by thin-layer chromatography, was as previously described (Pietrzykowska & Shugar, 1969), with the exception that the t.l.c. plates used were Silufol UV_{254} (Kavalier, Czechoslovakia). These plates were more advantageous since, due to their lower alkalinity, isomer II was more stable during chromatography, with resultant higher yields. The R_F values for the two isomeric photohydrates, using the same solvent system as previously (chloroform - methanol, 85:15, v/v), were identical with those on silica gel, i.e. 0.22 for isomer I and 0.67 for isomer II.

As in the case of isomer I, isomer II was eluted from the plates with methanol, brought to dryness, crystallized 3× from anhydrous ethanol, washed with ether and dried over P_2O_5.

In a typical preparation, 110 mg UdR was irradiated at a concentration of 10^{-3} M. Under these conditions, about 8% of the UdR is transformed to cyclobutane dimers. The final yields of the two isomeric photohydrates, in crystalline form, were 31 mg of isomer I and 10.4 mg of isomer II. Bearing in mind that the ratio of the two isomeric photohydrates in the irradiated solution is I/II=7/3 (Pietrzykowska & Shugar,

1969), the yields of the crystalline products are 43% for isomer I and 33% for isomer II.

When the isolated crystals of isomer II, in the form of elongated parallelopipeds, were heated on a microscope hot stage, they "melted" sharply at 126°C (uncorrected). This "melting" was accompanied by liberation of water to form a concentrated solution of UdR. It should be recalled that isomer I, which crystallizes in the form of platelets, "melts" out in a similar manner, but at 56°C (Pietrzykowska & Shugar, 1969).

Kinetics of dehydration of UdR·H_2O *isomers in neutral medium.* It was previously shown that the mechanisms of acid and alkaline dehydration for the isomeric photohydrates are different (Pietrzykowska & Shugar, 1969). We now proceed to an examination of the kinetics of dehydration of the two isomers under varying conditions.

The kinetics of thermal dehydration (80°C) at neutral pH of the two isomeric photohydrates, in H_2O and in D_2O, are shown in Fig. 1, from which it will be noted

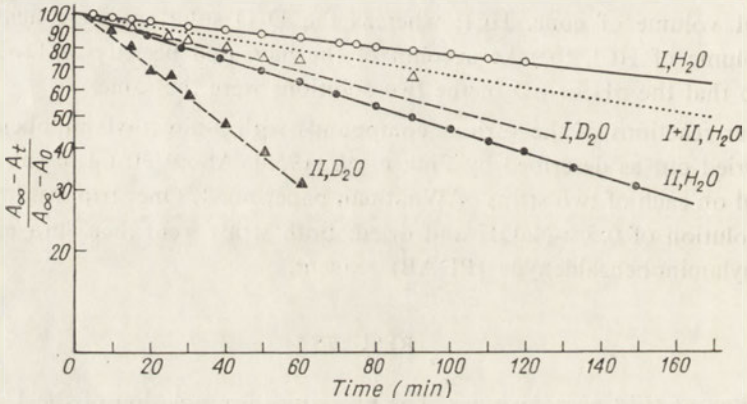


Fig. 1. Kinetics of thermal reversal of UdR·H_2O isomers I and II to UdR in neutral aqueous medium in H_2O and D_2O at 80°C, as indicated in the figure; (I+II), mixture of non-fractionated isomers from irradiated UdR in H_2O. A_0 is the absorbance of the solution at time 0, A_t the value at time t, and A_∞ upon completion of the dehydration reaction.

that isomer II is more labile, its rate constant for reversal to UdR being fourfold higher than for isomer I. The time-course of dehydration for both isomers is first-order, as might be anticipated, the rate constants in H_2O being 12.0×10^{-5} sec^{-1} and 2.8×10^{-5} sec^{-1} for isomers II and I, respectively. It will also be seen, as earlier reported (Wierzchowski & Shugar, 1961), that the corresponding rate constants in D_2O are higher, the isotope effect k_{H_2O}/k_{D_2O} being 1.6 for isomer I and 2.0 for isomer II.

When a solution of UdR was irradiated at 254 nm to the extent of 90% photohydration and then subjected to thermal dehydration in H_2O under the same conditions as above, the rate of dehydration was intermediate between those for the two isomers (Fig. 1), with a rate constant of 7.6×10^{-5} sec^{-1}, corresponding to the rate

constant for a mixture of 70% of isomer I and 30% isomer II. This distribution of isomers is precisely that previously found by direct chromatographic separation of the two isomeric photohydrates of UdR (Pietrzykowska & Shugar, 1969).

Kinetics of dehydration in acid medium. A comparison of the kinetics of dehydration of the UdR·H_2O isomers in acid medium (0.1 M-HCl, pH 1) at 40°C (Fig. 2) provided additional evidence for the previous observation (Pietrzykowska &

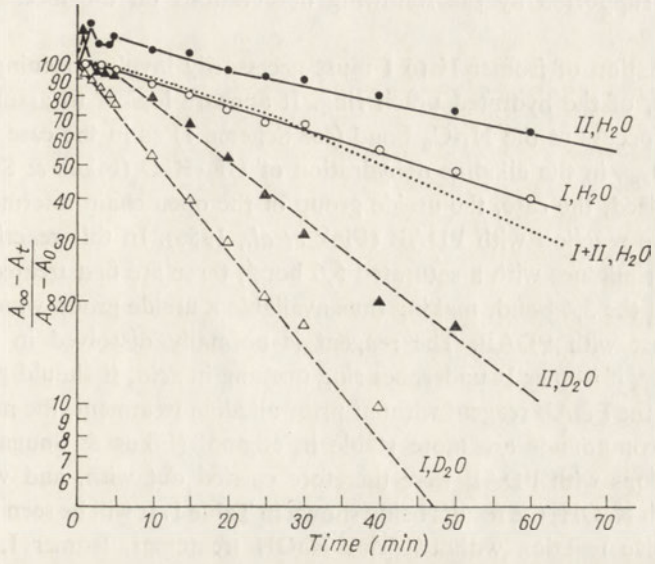


Fig. 2. Reversal of UdR·H_2O isomers I and II to UdR in H_2O and D_2O at pH~1 (0.1 M-HCl) and 40°C. Symbols as in Fig. 1.

Shugar, 1969) that the mechanism of dehydration of isomer II in acid medium involves prior acid-catalysed transformation of isomer II to isomer I. This is clearly reflected by the initial instantaneous decrease in residual absorption of the isomeric photohydrate II at 262 nm, following which there are small increases and decreases in absorption (Fig. 2) and, eventually, after about 5 min heating, a continual increase in absorbance of the absorption maximum at 262 nm due to regeneration of UdR. No such complex changes were observed in the course of dehydration of isomer I, although the rate constant for this isomer was somewhat lower during the initial 5 min as compared to the subsequent course of the reaction.

The rate constant for dehydration of the two photohydrate isomers in acid medium in D_2O (pD=1) at 40°C were also higher than in H_2O under analogous conditions. The isotope effects were, in fact, appreciably higher than for thermal dehydration in neutral medium, and amounted to 4.0 for isomer I and 5.0 for isomer II. Furthermore, in D_2O the initial portion of the rate curve for isomer II exhibited the same anomalies as in H_2O, corresponding to initial transformation of II to I, but this process was faster in D_2O. Finally, the rate curves for dehydration of an irradiated solution of UdR, containing a mixture of the two isomers, corresponded in H_2O and D_2O to those for a mixture of 70% of isomer I and 30% of isomer

II. The magnitudes of the isotope effects are quantitatively similar to those reported in an earlier investigation on the photohydrates of uridine (Wierzchowski & Shugar, 1961).

Acid-catalysed transformation of isomer II to isomer I. The evidence previously presented (Pietrzykowska & Shugar, 1969), and that cited in the previous section, for the acid-catalysed dehydration of isomer II *via* its initial transformation to isomer I, is further supported by the following observations on the mechanism of this reaction.

Transformation of isomer II to I must necessarily involve opening, and subsequent closure, of the hydrated uracil ring. It appears logical to assume that such ring opening occurs at the N_1-C_6 bond (see Scheme 1) as in the case of formation of the $interm_{290}$ in the alkaline dehydration of UR·H_2O (Fikus & Shugar, 1966). If such is, indeed, the case, the ureide group of the open chain intermediate should give a positive reaction with PDAB (Fink *et al.*, 1956). In this reaction, normally applied to pyrimidines with a saturated 5,6 bond, these are first treated with strong alkali to open the 3,4 bond, making thus available a ureide group which gives a coloured product with PDAB. The reagent is normally dissolved in 0.5 M-HCl in ethanol; hence, if isomer II undergoes ring opening in acid, it should give a positive reaction with the PDAB reagent without prior alkaline treatment, the more so in that ring-opened compounds are more stable in ethanol (Fikus & Shugar, 1966).

The reactions with PDAB were therefore carried out with, and without, prior treatment with NaOH, the results being shown in Table 1. It will be seen that isomer II gives a positive reaction without prior NaOH treatment. Isomer I, by contrast, gives a positive reaction only after treatment with NaOH, like 5,6-dihydro derivatives of uracil and thymine (Fink *et al.*, 1956). A positive reaction without prior

Table 1

Reaction with p*-dimethylaminobenzaldehyde of the isomeric photohydrates of UdR*

The reaction was carried out as described by Fink *et al.* (1956): about 30 µg of product was deposited in the form of a spot on Whatman paper no. 1, in duplicate. One spot was sprayed with the PDAB reagent, the other with NaOH and then with the PDAB reagent.

Compound	Reaction with PDAB reagent	
	Without prior NaOH treatment	With prior NaOH treatment
UdR	—	—
UdR·H_2O isomer I	—	+
UdR·H_2O isomer II	+	—
UdR·H_2	—	+
$Interm_{290}$*	+	—**

* $Interm_{290}$ formed during alkaline reversal of isomer I, was obtained by treatment of UdR·H_2O-I in ammoniacal MeOH for 10 min, followed by neutralization with HCl to pH about 6. Under these conditions the intermediate is relatively stable (Fikus & Shugar, 1966).

** From Fikus & Shugar (1966).

NaOH treatment is also given by $interm_{290}$ formed during alkaline dehydration of isomer I. As regards the negative reaction of II following NaOH treatment, this is due to its rapid alkaline dehydration to the parent UdR which, of course, does not react with PDAB. This also explains the negative reaction previously reported for $interm_{290}$ following prior NaOH treatment (Fikus & Shugar, 1966).

The foregoing observations consequently support the other evidence for the acid--catalysed conversion of isomer II to isomer I *via* ring cleavage at the position N_1-C_6 as for the $interm_{290}$ formed during the alkaline dehydration of isomer I.

Stability of UdR·H_2O *isomers as a function of pH.* The stability of the two isomeric photohydrates was examined over the pH range 1 - 11.3, using the following 0.01 M buffer systems: pH 1.0 - 4.8, citrate; pH 3.6 - 5.4, acetate; pH 5.4 - 6.4, citrate-acetate; pH 7.2 - 8.0, Tris; pH 8.1 - 11.3, ammonium.

The rates of dehydration at various pH values were measured at room temperature, and were followed by monitoring the increase in absorption at 262 nm over the pH range 1 - 11.3 for isomer II and 1 - 8.1 for isomer I. At pH values above 8.1 the rate of transformation of isomer I to the intermediate was followed by measuring the increase in absorption with time at 290 nm.

For calculations of rate constants, the values of $(A_\infty - A_t)$ were obtained in the pH range 1 - 8 by complete reversal to UdR at pH 0 (i.e. 1 M-HCl) following prior reversal in buffer at a given pH. In the pH range 8.1 - 11.3, the latter pH was employed for complete conversion to the parent UdR.

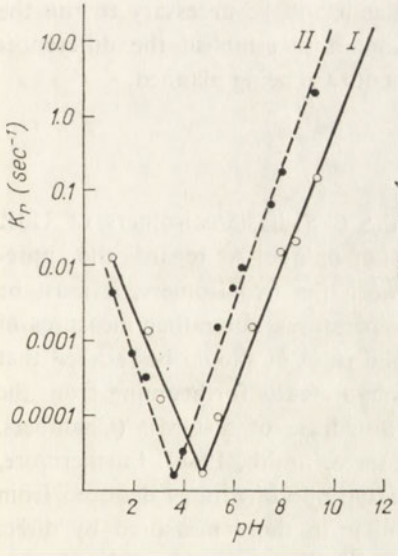


Fig. 3

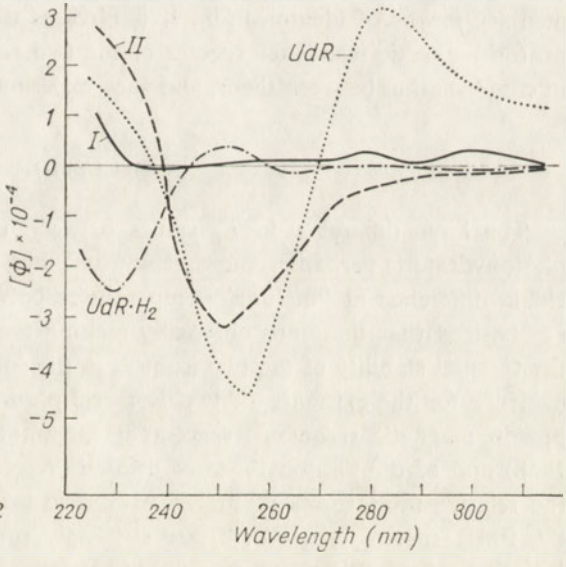


Fig. 4

Fig. 3. Stability of isomeric UdR photohydrates in aqueous medium at room temperature (20°C) as a function of pH. For further details see text; (——) isomer I; (— — —) isomer II. K_r is the rate constant for dehydration at a given pH.

Fig. 4. O.r.d. spectra of UdR·H_2O isomers I and II, compared with the spectra for UdR and UdR·H_2.

Figure 3 exhibits the pH-dependent rate constants for reversal of UdR·H_2O to UdR, or, at alkaline pH, for transformation of isomer I to the $interm_{290}$. It will be seen that isomer II exhibits maximum stability at pH 3.6, whereas isomer I is most stable at pH 4.8, the curve for isomer II being displaced by about 1.5 pH units relative to that for isomer I.

O.r.d. spectra of UdR·H_2O *diastereoisomers.* The o.r.d. spectra of the two isomeric photohydrates were recorded in aqueous medium at neutral pH, together with those for the parent UdR and the 5,6-dihydro derivative of the latter, i.e. UdR·H_2. The results are presented in Fig. 4. The very low rotatory dispersion for isomer I is somewhat surprising and rather difficult of interpretation; it should, however, be noted that at shorter wavelengths there is probably a positive band with a maximum at or below 220 nm as compared with the pronounced negative band exhibited by UdR·H_2 at 230 nm. Unfortunately technical limitations of the equipment itself made it impossible to extend measurements below 225 nm. By contrast, isomer II exhibits a relatively intense negative band at about 250 nm, where UdR·H_2 exhibits a weak positive band. It is also of some interest that the intensity of the negative band for isomer II is comparable to that for the negative band of UdR itself. Furthermore, on dehydration of isomer II, the resulting UdR spectrum coincided with that for authentic UdR.

In the absence of further information regarding the conformations of the two isomeric photohydrates, it would be premature to attempt to draw any specific conclusions from these o.r.d. spectra, which for the moment serve only as a supplementary means of identification. It is obvious that it will be necessary to run the proton magnetic resonance spectra of the two isomers to establish the differences in conformation between them, and such experiments are being planned.

DISCUSSION

The large difference in R_F values between the two diastereoisomers of UdR photohydrate is perhaps somewhat unusual, but not unique. As regards the appreciable difference in "melting" temperatures between the two isomers, it must be emphasized that these are not really melting temperatures but rather measures of the thermal stability of the two isomers in the solid state. It should be recalled that evidence for the existence of two isomeric photohydrates is forthcoming from the proton magnetic resonance spectra of the photohydrate of 5′-UMP (Chambers, 1968) and of the photohydrate of uridine (Wechter & Smith, 1968). Furthermore, the relative proportions of the two isomeric uridine photohydrates deduced from the PMR spectra, viz. 60 : 40 are strikingly similar to those measured by direct isolation, 65:35 (Pietrzykowska & Shugar, 1969). Finally, the acid-catalysed conversion of isomer II to isomer I; the quantitative agreement between the kinetics for dehydration of the individual isomers of UdR·H_2O with those for an unfractionated mixture; and the quantitative conversion of each of the isomers to the parent UdR with elimination of water, are all consistent with the two photohydrates being isomeric. The possibility that the water hydroxyl may be located on the C_5 of one

of the isomers is excluded by the fact that both undergo rapid dehydration in alkaline medium to regenerate UdR; under these conditions a derivative with the OH on position 5 would undergo ring opening of the N_3-C_4 bond (Moore & Thomson, 1956).

The marked difference in o.r.d. spectra between the two isomeric photohydrates underlines the appreciable difference in configuration, but does not, unfortunately, provide any real clue as to the nature of these differences.

It would be of interest to establish whether the products of photohydration of cytosine nucleosides also include the two diastereoisomers. Unfortunately the ease with which cytosine photohydrates eliminate water in the dark probably excludes the possibility of direct separation of such isomers. Indirect evidence for formation of two diastereoisomers has been obtained by chemical degradation (Miller & Cerutti, 1968). However, a conceivably practical procedure for their isolation might be based on the use of N^4-alkylcytosine nucleosides, the photohydrates of which are remarkably stable (Fikus *et al.*, 1962).

In a study of the kinetics of dehydration of uridine photohydrate, and some of its derivatives, at elevated temperatures, Logan & Whitmore (1966) observed a shoulder in the initial portion of the kinetic curves for all the derivatives. This was ascribed by them to the lag in dehydration rate due to the time required for the solution to attain the desired temperature. The present findings demonstrate, however, that at least a portion of the initial lag in dehydration rate may be due to other factors, as outlined above. This is further supported by the results shown in Fig. 2, where the temperature employed for dehydration was only 40°C, hence not much above ambient temperature, but showing clearly the initial "lag" phase. It should, furthermore, be noted that these authors employed Tris buffers, the temperature coefficients for which are very large (Good *et al.*, 1966), so that appreciable changes of pH occurred during heating of the solutions to the required temperatures.

Although the biological significance of pyrimidine photohydrates has been less extensively investigated than that of pyrimidine photodimers, the lethal and genetic effects resulting from photohydration of pyrimidine residues in nucleic acids have been well substantiated (Carpenter & Kleczkowski, 1969; Tao *et al.*, 1969; Remsen *et al.*, 1971; Mattern *et al.*, 1972). In the case of cytosine photohydration, which proceeds extensively in both synthetic and natural polynucleotides (Wierzchowski & Shugar, 1962; Johns *et al.*, 1965; Grossman & Rodgers, 1968), the accompanying deamination of cytosine photohydrate to uracil photohydrate (Johns *et al.*, 1965) would lead naturally to base-pair transitions.

Furthermore, studies on the mechanism of deuterium exchange at the 5-position of uracil and cytosine photohydrates during the dark dehydration reaction (Chambers, 1968; Wechter & Smith, 1968) have hitherto ignored the presence of two isomeric photohydrates, a factor which must of necessity be taken into account. In fact, the mechanism for dehydration of Urd·H_2O advanced by Wechter & Smith (1968) is in disagreement with that proposed earlier by Wierzchowski & Shugar (1961). The former authors attribute this inconsistency in part to errors in measurements of the isotope effects for dehydration of the uridine photohydrates prepared in

heavy water (Wierzchowski & Shugar, 1961). We have now repeated these measurements (Wierzchowski & Shugar, unpublished) and find that they agree with those earlier reported. It is consequently the mechanism proposed by Wechter & Smith (1968) which requires reexamination, the more so in that the studies of DeBoer & Johns (1970) demonstrated that up to 15% of the C_5 proton of the cytosine ring exchange with the solvent during the irradiation step leading to the photohydrate. This would necessarily lead to appreciable errors in studies on the kinetics of exchange of the C_5 proton during dark dehydration, and in the conclusions derived therefrom.

The complexity of the mechanism of dehydration is further attested to by the observation previously reported (Pietrzykowska & Shugar, 1970), viz. that the rate of dehydration is appreciably affected by the anomeric configuration at $C_{1'}$. For example the acid-catalysed dehydration of the α-anomer of $UdR \cdot H_2O$ (containing a 1:1 mixture of the isomeric photohydrates) is approximately 8 - 10 times faster than for the corresponding β-anomer.

We are indebted to Dr. Andrzej Rabczenko for running the o.r.d. spectra, to Dr. Zofia Zarębska for discussions and to Mrs. Maria Żylonis for technical assistance.

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WŁASNOŚCI IZOMERÓW PRZESTRZENNYCH FOTOHYDRATÓW NUKLEOZYDÓW URACYLU

Streszczenie

Izolowano metodą chromatografii cienkowarstwowej dwa izomery fotohydratu 2′-dezoksyurydyny i uzyskano je w formie krystalicznej. Badano kinetykę odłączania cząsteczki wody z obu izomerów w środowisku kwaśnym i obojętnym w podwyższonej temperaturze. Wyniki badań nad kinetyką rewersji izomeru II w środowisku kwaśnym potwierdziły wcześniejsze obserwacje, że w tych warunkach odłączenie cząsteczki wody z izomeru II następuje po uprzednim przekształceniu izomeru II w izomer I. Przekształcenie to zachodzi najprawdopodobniej na drodze otwarcia wiązania 1 - 6 pierścienia pirymidynowego.

Received 14 December 1973.

ACTA BIOCHIMICA POLONICA

Vol. 21 1974 No. 2

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INTERRELATIONSHIP BETWEEN PHOSPHOENOLPYRUVATE AND CITRULLINE SYNTHESIS IN GUINEA PIG LIVER MITOCHONDRIA

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1. In coupled mitochondria, very little phosphoenolpyruvate formation occurred with glutamine and proline as substrate, similarly as it was shown with glutamate (Bryła *et al.*, *Biochim. Biophys. Acta*, 1973, **314**, 411 - 417) since aspartate aminotransferase has a much higher affinity towards intramitochondrial oxaloacetate than phosphopyruvate carboxylase does. Inhibition of aspartate synthesis by aminooxyacetate resulted in 3 - 6 fold increase in the phosphoenolpyruvate formation with both glutamate and glutamine as precursors of oxaloacetate, but was without effect when proline was used as the substrate. 2. In mitochondria incubated in state 3 with glutamate, proline or glutamate + glutamine the rates of citrulline formation were low due to the small production of ammonia. This limitation could be overcome on using glutamine as the substrate. 3. GTP provided by substrate level phosphorylation was utilized more efficiently for phosphoenolpyruvate synthesis than that produced from exogenous ATP *via* nucleoside diphosphate kinase. 4. In the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and oligomycin the rate of citrulline synthesis was dependent upon the levels of intramitochondrial ATP. Under conditions resulting in a decrease of ATP down to 2.2 - 2.5 nmol/mg protein, citrulline formation was almost completely inhibited. Restoration was observed on addition of exogenous ATP. 5. The results suggest that under conditions of low energy generation there is a competition for energy between synthesis of phosphoenolpyruvate and citrulline.

Guinea pig liver mitochondria are very useful for studies on the interrelationship between the first steps of gluconeogenesis and urea production since in this species, like in humans, phosphoenolpyruvate (Ballard & Hanson, 1968; Böttger *et al.*, 1969) as well as citrulline (Cohen & Hayano, 1948; Leuthardt & Müller, 1948), are produced intramitochondrially. The rate of phosphoenolpyruvate synthesis in guinea pig liver mitochondria was investigated in the presence of tricarboxylic acid cycle intermediates as precursors of oxaloacetate (Garber & Ballard, 1969, 1970; Garber & Salganicoff, 1973; Bryła *et al.*, 1973a). However, citrulline formation

has been extensively studied only in rat liver mitochondria (Charles & Tager, 1967; Charles & van den Bergh, 1967; Charles *et al.*, 1967; Graafmans *et al.*, 1968; Tager, 1969) which do not contain phosphopyruvate carboxylase (EC 4.1.1.32) (Böttger *et al.*, 1969).

In the present work, phosphoenolpyruvate[1] and citrulline formation was studied in guinea pig liver mitochondria incubated under two metabolic conditions of different ATP levels. Glutamate, glutamine and proline, which are metabolized intramitochondrially, were used both as energy source and precursors of oxaloacetate and ammonia for phosphoenolpyruvate and citrulline synthesis, respectively. The results suggest that under conditions of low energy generation there is a competition for energy between synthesis of phosphoenolpyruvate and citrulline. Data on glutamate as a substrate have been reported in a preliminary form (Bryła *et al.*, 1973b,c).

MATERIALS AND METHODS

Incubation of mitochondria. Mitochondria were prepared as described by Schneider & Hogeboom (1950) from the livers of male albino guinea pigs weighing 250 - 300 g; the medium contained 225 mM-mannitol, 75 mM-sucrose and 0.1 mM-EDTA. The mitochondria (3 - 4 mg protein/ml) were incubated in 15 mM-KCl - 2 mM-EDTA - 5 mM-$MgCl_2$ - 40 mM-Tris-HCl buffer - 10 mM-ornithine - 30 mM-$KHCO_3$ and 20 mM-potassium phosphate buffer, with the additions indicated in the legends to Tables and Figures. The final pH was 7.4. The amino acid substrates were added at concentrations which did not limit their oxidation in mitochondria, i.e.: 10 mM-glutamate, 15 mM-proline and 30 mM-glutamine; when glutamate and glutamine were added together, their concentrations were 5 and 25 mM, respectively.

Mitochondria were incubated as described previously (Bryła *et al.*, 1973a) at 30°C under two different respiratory states: in state 3, on addition of 30 mM-glucose, 0.1 mM-ADP and hexokinase (EC 2.7.1.1), 2 - 5 units/ml; and in uncoupled state in the presence of 0.2 - 0.5 μM-FCCP and oligomycin (1 μg/mg protein).

Assays of metabolites. Samples of 2 ml withdrawn from the incubation mixture at 5 min intervals (up to 20 min), were deproteinized on addition of 0.2 ml of 35% $HClO_4$, and neutralized. $KClO_4$, which precipitated in the cold, was then centrifuged off.

Phosphoenolpyruvate and aspartate were determined according to Czok & Lamprecht (1970) and Williamson & Corkey (1969), respectively. ATP was measured fluorimetrically (Williamson & Corkey, 1969), and citrulline colorimetrically by the method of Archibald (1944) as described by Charles *et al.* (1967). Oxygen consumption was followed in a thermostated vessel with the Clark-type oxygen electrode. Mitochondrial protein was measured by the biuret method (Cleland & Slater, 1953).

Materials. ADP, ATP and NADH were commercial preparations supplied by Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Aminooxyacetic acid was provided

[1] Abbreviations: PEP, phosphoenolpyruvate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; AOA, aminooxyacetate.

by Eastman Kodak Co. (Rochester, N. Y., U.S.A.). L-Proline, L-glutamine and L--ornithine were purchased from Reanal (Budapest, Hungary). FCCP was kindly supplied by Dr. Peter Heytler of the DuPont Chemical Co. (Wilmington, Del., U.S.A.).

Pyruvate kinase (EC 2.7.1.40), aspartate aminotransferase (EC 2.6.1.1), malate dehydrogenase (EC 1.1.1.37) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) used for assays of metabolites were prepared after Tietz & Ochoa (1962), Sizer & & Jenkins (1962), Englard & Siegel (1969) and Kuby & Noltmann (1966), respectively. $(NH_4)_2SO_4$-free hexokinase was from Koch-Light Lab. Ltd (Colnbrook, Bucks, England), and lactate dehydrogenase (EC 1.1.1.27) from Biomed (Kraków, Poland).

RESULTS

Oxidation of amino acids and synthesis of phosphoenolpyruvate and citrulline. The rates of oxygen uptake by mitochondria incubated with glutamine, glutamate, proline, or glutamine + glutamate, in state 3 or in the presence of uncoupler + oligomycin, are shown in Table 1. Oligomycin was added to inhibit dissipation of ATP due to ATPase activity (Lardy *et al.*, 1958).

Table 1

Oxygen uptake in mitochondria incubated with various amino acids in state 3 and in the presence of FCCP and oligomycin

Experimental conditions were as under Methods. Values shown are means of 4 separate experiments ±S.E.M.

Substrate	Oxygen uptake (natoms/min/mg protein)	
	state 3	FCCP+oligomycin
Glu	35.2±5.2	47.0±5.8
Glu(NH_2)	24.8±7.2	40.4±2.4
Pro	22.0±2.2	28.8±4.8
Glu+Glu(NH_2)	41.6±3.2	54.8±2.4

In mitochondria incubated in state 3 oxidation of glutamate and glutamine + glutamate was much higher than that of glutamine and proline. Addition of uncoupler + oligomycin resulted in stimulation of the mitochondrial respiration by about 40 - 60% with all substrates.

The rates of synthesis of phosphoenolpyruvate and citrulline do not follow those of oxygen consumption. Data on the synthesis of these two compounds presented below include the results obtained with glutamate, reported previously (Bryła *et al.*, 1973c). The results presented in Fig. 1 demonstrate that in mitochondria incubated in state 3 very little phosphoenolpyruvate formation occurred with any of the substrates. The highest citrulline production was observed in the presence of glutamate + glutamine. With glutamate, glutamine or proline added separately, citrulline synthesis was 2 - 4 times lower suggesting that ammonia production could be rate--limiting.

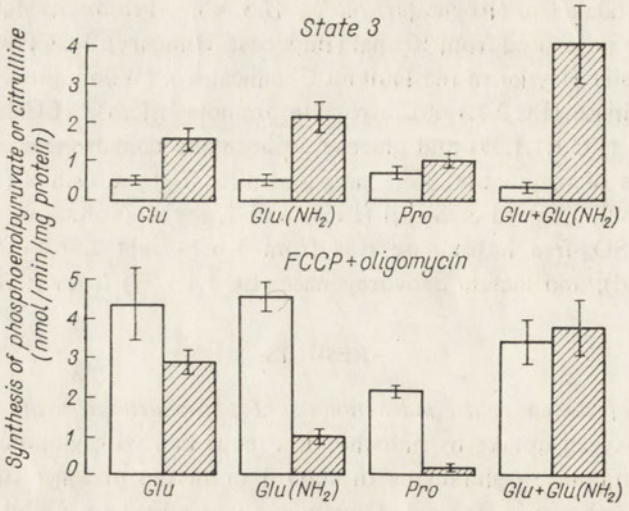

Fig. 1. Synthesis of phosphoenolpyruvate and citrulline in mitochondria incubated in state 3 and in the presence of FCCP + oligomycin. Values shown are means from 4 separate experiments ±S.E.M. (shown as vertical lines). Open bars, synthesis of phosphoenolpyruvate; strippled bars, synthesis of citrulline.

The addition of FCCP and oligomycin to the reaction medium resulted in the eight- to tenfold increase of phosphoenolpyruvate formation when glutamate and glutamine were substrates and in four- to fivefold stimulation in the presence of proline as the precursor of oxaloacetate. On the contrary, under uncoupled conditions citrulline synthesis was increased about twofold only with glutamate as a nitrogen donor. With glutamate + glutamine the rate of citrulline production was the same as in state 3, whereas on addition of either glutamine or proline it was markedly inhibited.

In order to explain these discrepancies between oxygen uptake and the rates of both phosphoenolpyruvate and citrulline synthesis, the two processes were studied at different levels of oxaloacetate, ammonia and energy.

The availability of oxaloacetate for aspartate and phosphoenolpyruvate synthesis. Mitochondria incubated in state 3 produce much higher amounts of aspartate than of phosphoenolpyruvate. This suggests that aspartate aminotransferase competes efficiently with phosphopyruvate carboxylase for oxaloacetate generated during oxidation of amino acids (Table 2). The highest rates of aspartate production were observed in the presence of glutamate alone and glutamate + glutamine. In agreement with the data on oxygen consumption (see Table 1) the rate of aspartate formation was much lower with glutamine or proline as substrate. Addition of aminooxyacetate, an inhibitor of aspartate aminotransferase (Hopper & Segal, 1962), resulted in complete inhibition of aspartate production and a concomitant three- to sixfold increase in phosphoenolpyruvate formation. In the presence of proline, phosphoenolpyruvate synthesis was not stimulated.

Table 2

Effect of aminooxyacetate on the synthesis of phosphoenolpyruvate and aspartate in state 3 and in the presence of FCCP + oligomycin

Experimental conditions as described under Methods; 0.1 mM-aminooxyacetate (AOA) was added where indicated. Values shown are means of 3 separate experiments, ±S.E.M.

Incubation conditions	Rate of synthesis (nmol/min/mg protein)			
	Control		+AOA	
	PEP	Asp	PEP	Asp
State 3 substrate:				
Glu	0.5±0.1	10.9±0.6	2.4±0.3	0
Glu(NH_2)	0.5±0.1	3.7±1.1	1.3±0.1	0
Pro	0.7±0.1	1.5±0.4	0.6±0.1	0
Glu+Glu(NH_2)	0.3±0.1	12.2±0.2	2.0±0.7	0
FCCP+oligomycin substrate:				
Glu	4.3±0.9	9.1±0.5	10.1±1.1	0
Glu(NH_2)	4.5±0.4	3.1±0.7	5.1±0.5	0
Pro	2.1±0.1	0.1±0.1	2.5±0.1	0
Glu+Glu(NH_2)	3.4±0.6	12.0±0.2	9.3±0.2	0

On addition of uncoupler and oligomycin the rates of phosphoenolpyruvate production were much higher than in state 3, although aspartate synthesis was practically unchanged (Table 2) with all the substrates used except proline. In the latter case, formation of aspartate was inhibited. The increase in phosphoenolpyruvate formation under uncoupled conditions was probably due to the fact that uncoupler stimulates oxidation of amino acids (see Table 1) by increasing the flux through the dehydrogenation pathway. When aminooxyacetate was included in the reaction mixture, phosphoenolpyruvate formation was doubled in the presence of glutamate and only slightly increased with glutamine or proline. This is in agreement with lower rates of oxygen consumption with these two latter substrates.

Sources of energy for phosphoenolpyruvate formation in uncoupled mitochondria. Under uncoupled conditions GTP required for phosphopyruvate carboxylase is generated *via* the substrate level phosphorylation. Since ATP can provide GTP *via* nucleoside diphosphate kinase (EC 2.7.4.6), the effect of exogenous ATP on the rate of phosphoenolpyruvate formation was studied. Data presented in Fig. 2 indicate that with glutamate and glutamine + glutamate, addition of ATP resulted in a 45% decrease of phosphoenolpyruvate production. Inhibition of this process by exogenous ATP was lower (about 20%) when glutamine or proline were used as substrates. The results indicate that in the presence of added ATP less GTP is utilized for phosphoenolpyruvate formation.

Ammonia and energy requirement for citrulline synthesis. Addition of aminooxyacetate to mitochondria in state 3, in contrast to phosphoenolpyruvate formation,

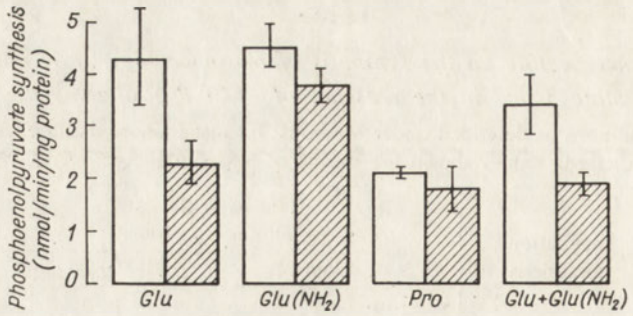

Fig. 2. Effect of exogenous ATP on phosphoenolpyruvate synthesis in mitochondria incubated with FCCP + oligomycin. Values shown are means from 3 experiments ±S.E.M. Citrulline synthesis in the absence (open bars) and in the presence (strippled bars) of 5 mM-ATP.

increased but slightly citrulline production with glutamate as substrate, and was completely ineffective with other substrates (Fig. 3). However, when ammonium chloride was included in the reaction mixture, citrulline synthesis was stimulated about fourfold with glutamate and about twofold with proline or glutamate + glutamine. This indicates that ammonia production is a limiting factor for the formation of citrulline. Since aminooxyacetate did not affect citrulline synthesis in the presence of glutamine it appears that production of ammonia by glutaminase

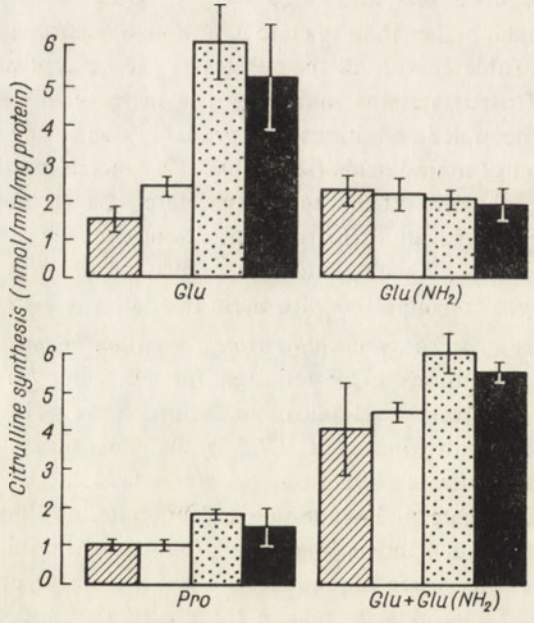

Fig. 3. Synthesis of citrulline in mitochondria incubated in state 3 in the presence of aminooxyacetate and/or ammonium chloride. Values shown are means from 3 experiments ± S.E.M. Strippled bars, no additions; open bars, with 0.1 mM-aminooxyacetate; dotted bars, with 10 mM-NH_4Cl; solid bars, with 0.1 mM-aminooxyacetate + 10 mM-NH_4Cl.

is sufficient to support citrulline formation when the ATP required for carbamoylphosphate synthase (EC 2.7.2.5) is generated during glutamine oxidation.

Under conditions favouring citrulline formation, glucose 6-phosphate synthesis decreased (Fig. 4). The lowest rate of glucose 6-phosphate synthesis was observed in the presence of aminooxyacetate added together with ammonium chloride because under these conditions energy was utilized for both citrulline and phosphoenolpyruvate synthesis.

As it is shown in Fig. 5, addition of aminooxyacetate to mitochondria incubated under uncoupled conditions resulted in a marked decrease of citrulline synthesis even in the presence of ammonium chloride. Moreover, ammonium chloride alone had no effect, indicating that ammonia production in the uncoupled mitochondria is not rate-limiting. These observations suggest that inhibition of citrulline formation on addition of aminooxyacetate might be due to a decreased generation of ATP *via* the nucleoside diphosphate kinase. In order to check this hypothesis, concentration of ATP was determined in the incubation mixtures (Fig. 6). With no additions made to the uncoupled mitochondria, ATP concentration was twice as high with glutamate or glutamate + glutamine as with glutamine alone. On addition of aminooxyacetate the ATP level was practically the same with all the substrates used, and almost equal to that measured in the presence of glutamine alone.

Similar values were also obtained when aminooxyacetate was added together with ammonium chloride. On the other hand, when ammonium chloride was included alternatively with glutamate or glutamate + glutamine (but not with glutamine), ATP levels were higher than those found in the presence of aminooxyacetate. The data presented show a correlation between citrulline formation and ATP level (see Fig. 5).

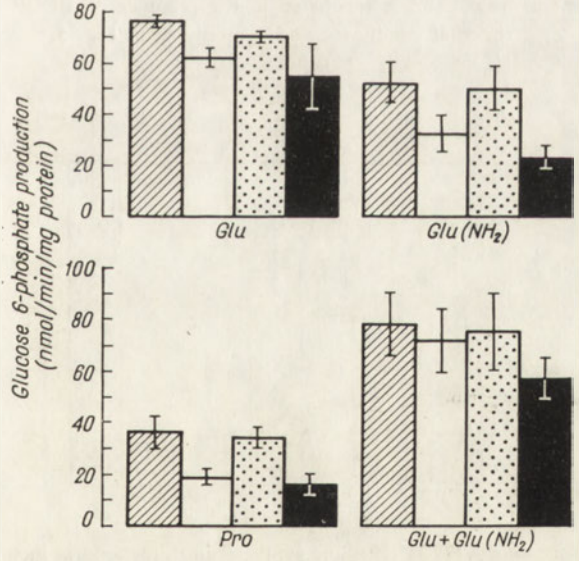

Fig. 4. Glucose 6-phosphate production in mitochondria incubated in state 3 in the presence of aminooxyacetate and/or ammonium chloride. Experimental conditions and symbols as in Fig. 3.

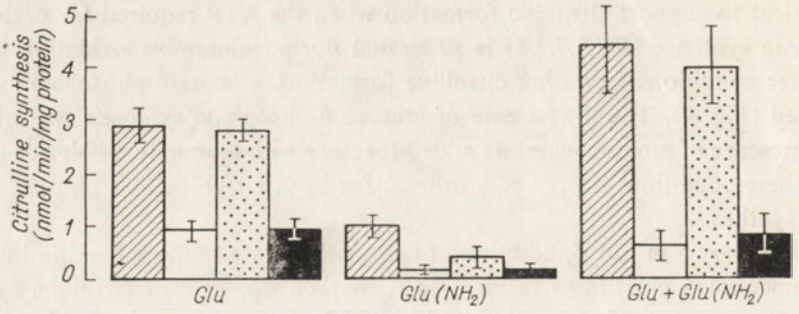

Fig. 5. Citrulline synthesis in mitochondria incubated in the presence of FCCP + oligomycin, with aminooxyacetate and/or ammonium chloride. Strippled bars, no additions; open bars, with 0.1 mM-aminooxyacetate; dotted bars, with 10 mM-NH_4Cl; solid bars, with 0.1 mM-aminooxyacetate + 10 mM-NH_4Cl.

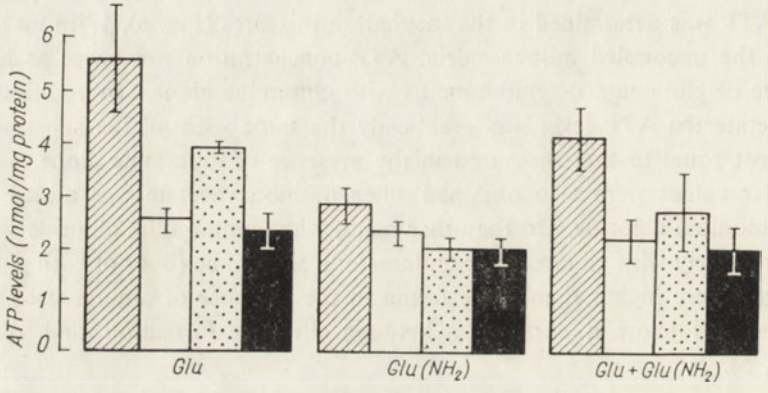

Fig. 6. ATP content in mitochondria incubated in the presence of FCCP + oligomycin. Experimental conditions and symbols as in Fig. 5.

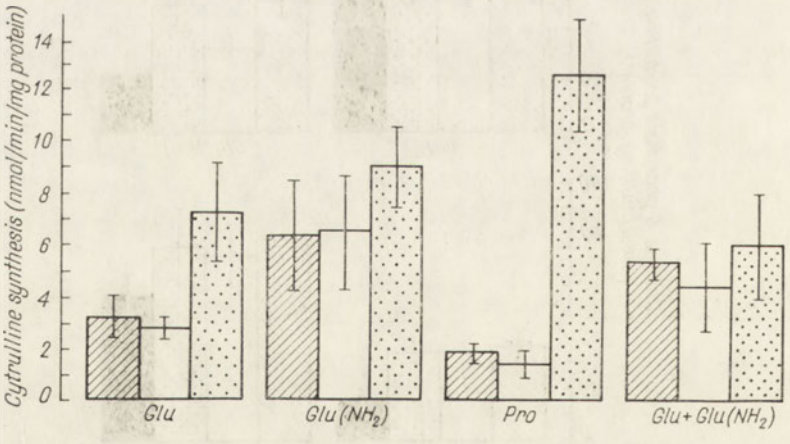

Fig. 7. Effect of exogenous ATP on citrulline synthesis in mitochondria incubated in the presence of FCCP + oligomycin. The reaction mixture contained 5 mM-ATP. Other experimental conditions and symbols as in Fig. 5.

Since the data in Fig. 5 and 6 suggest that under uncoupled conditions generation of ATP during oxidation of amino acids could be the rate-limiting factor, the rate of citrulline synthesis was studied in the presence of 5 mM-ATP (Fig. 7). When no additions were made to the mitochondria incubated with glutamate, citrulline production was similar to that determined in the absence of ATP (see Fig. 1). On the other hand, citrulline synthesis was stimulated on addition of ATP, about sixfold with glutamine, even stronger with proline, but to a much lower extent with glutamate + glutamine. This indicates limitation of citrulline synthesis by ATP during oxidation of glutamine and proline.

Addition of aminooxyacetate in the presence of ATP practically did not affect citrulline synthesis with any of the substrates studied. On the other hand, ammonium chloride in the presence of ATP stimulated further citrulline formation with all the substrates used. This effect was, however, much smaller when glutamate + glutamine were used as precursors of ammonia. Since the increase of citrulline synthesis on addition of ammonium chloride was much higher with proline (which is poorly oxidized in mitochondria) than with glutamate + glutamine it seems likely that in regulation of citrulline formation a transport phenomenon is also involved.

DISCUSSION

Data presented in this paper permit an evaluation of the various factors contributing to the regulation of amino acid utilization in intact mitochondria respiring in different metabolic conditions. These are: (i) availability of oxaloacetate for phosphoenolpyruvate formation, (ii) generation of ammonia for citrulline production, (iii) competition for energy between phosphoenolpyruvate and citrulline synthesis.

Oxaloacetate availability for phosphoenolpyruvate synthesis. It could be expected that under our experimental conditions total oxaloacetate would be bound to malate dehydrogenase, aspartate aminotransferase and phosphopyruvate carboxylase. The content of oxaloacetate in guinea pig liver mitochondria incubated in the active state is 10 - 13 μM (Bryła, Fukami and Williamson, unpublished). K_m values for malate dehydrogenase, aspartate aminotransferase and phosphopyruvate carboxylase are equal to 4×10^{-5} M (Grimm & Doherty, 1961), 8.8×10^{-5} M (Henson & Cleland, 1964) and 2×10^{-5} - 9×10^{-6} M (Ballard, 1970), respectively, i.e. they are above the measured levels of intramitochondrial oxaloacetate. Thus, oxaloacetate availability can be a limiting factor for phosphoenolpyruvate synthesis especially when the transamination pathway of glutamate is active.

Inhibition of phosphoenolpyruvate formation in coupled mitochondria is due to a much higher affinity of aspartate aminotransferase for intramitochondrial oxaloacetate as compared with phosphopyruvate carboxylase. This indicates that glutamate oxidation under these conditions proceeds mainly *via* the transamination pathway (De Haan *et al.*, 1967). Although uncoupler is known to switch the transamination of glutamate to dehydrogenation (De Haan *et al.*, 1967), in our hands, however, the formation of aspartate was only about 10% lower than that measured in state 3. Although it has been proposed that the transport of aspartate is energy-

-dependent (LaNoue & Bryła, 1971), the efflux of aspartate from mitochondria incubated with glutamate or glutamine could presumably proceed, since ATP levels in the reaction medium containing FCCP + oligomycin were not lower than 2 nmol/mg protein (Fig. 6). The only exception was the lack of aspartate formation in the presence of proline, poorly oxidized in mitochondria. In this case energy generation could limit the transport of aspartate.

Under uncoupled conditions glutamate generated from amino acids is oxidized *via* both the transamination and dehydrogenation pathways resulting in an increase of phosphoenolpyruvate synthesis. This stimulation could be also due to a higher intramitochondrial oxaloacetate concentration available for phosphopyruvate carboxylase since in the uncoupled state the NADH level is lower than in state 3 (Bryła, Fukami and Williamson, unpublished). The maximal phosphoenolpyruvate synthesis (up to about 10 nmol/min/mg protein, i.e. 0.6 μmol/min/g liver wet wt.) takes place on complete inhibition of aspartate aminotransferase, as shown with aminooxyacetate. This rate of phosphoenolpyruvate formation seems to be sufficient, since according to Garber & Ballard (1970) production of approximately 1 μmol/min/g wet wt. of tissue is required to support the gluconeogenic pathway.

Ammonia generation for citrulline production. The interrelationship between glutamine metabolism and citrulline synthesis implies complex regulatory mechanisms in nitrogen metabolism in mitochondria. Ammonia generated *via* glutamine deamidation in mitochondria incubated in state 3 is sufficient to support citrulline formation in urea synthesis, when energy is provided exclusively from glutamine oxidation. However, low permeability of liver mitochondria to glutamine might be a limiting factor in production of ammonia. On the other hand, mitochondria show a high ability to metabolize glutamine and respond readily to its increased concentration over a wide range (Blackburn & Hird, 1972).

According to Papa *et al.* (1966) and De Haan *et al.* (1967) deamination of glutamate in the coupled mitochondria is very low probably due to the inhibition of glutamate dehydrogenase by a high $NADPH/NADP^+$ ratio, and is stimulated on addition of uncoupler. The K_m for ammonia was found to be about 0.5 mM (Bryła *et al.*, 1973b,c). Assuming that ammonia penetrates easily through the mitochondrial membrane (Chappell & Crofts, 1966), one can calculate that concentration of this compound would not exceed 0.2 - 0.4 mM and is doubled after incubation of the uncoupled mitochondria for 20 min with glutamate and glutamine. Thus, it may be concluded that ammonia production might limit the rate of citrulline synthesis in the coupled mitochondria.

Data presented by Kovacevic (1971) suggest, however, that under physiological conditions the transamination pathway does not predominate in the oxidation of glutamate, as the rapid formation of acetyl-CoA keeps the oxaloacetate concentration too low for substantial utilization by aspartate aminotransferase. This can be the case during starvation when concentration of fatty acids is considerably increased.

Sources of energy for phosphoenolpyruvate and citrulline synthesis. GTP required for phosphoenolpyruvate formation can be generated either *via* the substrate level phosphorylation or from ATP *via* nucleoside diphosphate kinase. However, in agree-

ment with our previous studies (Bryła *et al.*, 1973a) the results presented in this paper do not support the suggestion that the energy requirement for the mitochondrial phosphoenolpyruvate production could be met by transphosphorylation from the ATP pool (Garber & Hanson, 1971a,b). On the contrary, the addition of exogenous ATP to uncoupled mitochondria resulted in a marked decrease of phosphoenolpyruvate formation (Fig. 2).

In coupled mitochondria ammonia production rather than energy generation seems to be a limiting factor for citrulline synthesis. On the other hand, when substrate phosphorylation is the only energy source, under conditions favoring phosphoenolpyruvate formation, citrulline production is inhibited due to a decreased generation of ATP. This conclusion is also supported by the following observations: (i) no inhibition of citrulline synthesis occurred when exogenous ATP was added (Fig. 7); (ii) the K_m for ATP is rather low, about 2.1 mM (Bryła *et al.*, 1973b,c); (iii) the highest amounts of citrulline were produced in rotenone-inhibited mitochondria when ATP and ammonium chloride were used as energy source and ammonia donor, respectively (Graafmans *et al.*, 1968; Bryła *et al.*, 1973b,c) Thus, one might conclude that high rates of citrulline synthesis are maintained when energy is produced *via* both substrate level phosphorylation and oxidative phosphorylation. When the substrate level phosphorylation is the only energy source, phosphopyruvate carboxylase shows a much higher affinity than the nucleoside diphosphate kinase for the intramitochondrial GTP, resulting in the inhibition of citrulline formation. This is likely in view of the observation that elevated ADP levels inhibit the conversion of GTP to ATP by nucleoside diphosphate kinase (Goffeau *et al.*, 1967; Colomb *et al.*, 1969).

On the basis of the results presented it may be concluded that under physiological conditions utilization of amino acids for phosphoenolpyruvate and citrulline synthesis can proceed under conditions of high generation of both oxaloacetate and energy.

We are very grateful to Professor John R. Williamson for making available the fluorometer for ATP measurements and to Dr. P. Heytler for the gift of FCCP. The excellent technical assistance of Mr. Z. Fraś is acknowledged.

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WSPÓŁZALEŻNOŚĆ SYNTEZY FOSFOENOLOPIROGRONIANU I CYTRULINY W MITOCHONDRIACH ŚWINKI MORSKIEJ

Streszczenie

1. Analogicznie jak wykazano poprzednio dla glutaminianu (Bryła i wsp., *Biochim. Biophys. Acta*, 1973, **314**, 411 - 417), w mitochondriach sprzężonych inkubowanych z glutaminą i proliną jako substratami obserwowano nieznaczną syntezę fosfoenolopirogronianu, ponieważ szczawiooctan jest zużywany głównie w reakcji katalizowanej przez aminotransferazę asparaginową. Z glutami-

nianem i glutaminą jako substratami zahamowanie syntezy asparaginianu przez aminooksyoctan powodowało 3 - 6 krotny wzrost szybkości wytwarzania fosfoenolopirogronianu.

2. W mitochondriach inkubowanych w stanie 3 z glutaminą, proliną lub glutaminianem i glutaminą łącznie, synteza cytruliny jest niska wskutek niewielkiej szybkości wytwarzania amoniaku. Natomiast w obecności glutaminy jako substratu ilość amoniaku powstająca w reakcji katalizowanej przez glutaminazę nie ogranicza syntezy cytruliny.

3. Synteza fosfoenolopirogronianu jest większa, gdy GTP powstaje w procesie fosforylacji substratowej. Szybkość wytwarzania GTP z egzogennego ATP w wyniku działania kinazy nukleozydodwufosforanowej nie jest wystarczająca dla zachowania maksymalnej syntezy fosfoenolopirogronianu.

4. Po dodaniu FCCP i oligomycyny szybkość syntezy cytruliny zależy od poziomu wewnątrzmitochondrialnego ATP. Spadek ilości ATP w środowisku reakcyjnym do 2,2 - 2,5 nmola/mg białka powoduje prawie całkowite zahamowanie syntezy cytruliny, natomiast dodanie ATP przywraca syntezę tego aminokwasu.

5. Wyniki wskazują na istnienie współzawodnictwa o energię między syntezą fosfoenolopirogronianu i cytruliny.

Received 19 December, 1973.

ACTA BIOCHIMICA POLONICA

Vol. 21 1974 No. 2

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ANABOLIC AND CATABOLIC ROUTES OF ARGININE METABOLISM IN *B. SUBTILIS* PRODUCING BACITRACIN

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1. Arginase and ornithine carbamoyltransferase are induced in *B. subtilis* during forespore formation, and ornithine δ-aminotransferase during development of spore cortex or coat. 2. The activities of enzymes metabolizing ornithine in anabolic and catabolic routes of arginine metabolism are negatively correlated with the bacitracin formation, while the activity of arginase is not related to the synthesis of antibiotic. 3. Ornithine limits biosynthesis of bacitracin in the mutant with high activity of ornithine δ-aminotransferase but not in the strains with enzymic block in the δ-transamination step. 4. The size of the intracellular pools of amino acids and ornithine does not depend on the extracellular proteolytic activity of the mutants, nor on the effect of factors stimulating (glyoxylate) or inhibiting (acetate) the proteolytic activity.

Arginine occurs in polypeptide antibiotics rarely, however it serves as a general donor of amidine group in biosynthesis of such antibiotics as streptomycin and viomycin (Walker & Walker, 1967; Paśś & Raczyńska-Bojanowska, 1969). Besides, ornithine is a very common constituent of microbial peptides (Katz, 1970), among them of bacitracin. It has been recently suggested that the non-protein amino acid constituents, e.g. ornithine and diaminobutyrate, regulate the formation of polypeptide antibiotics by inhibiting aminoacyl-tRNA synthetases which compete for amino acids with the enzymes catalysing formation of antibiotics (Ito *et al.*, 1970).

In the present paper it has been shown that biosynthesis of bacitracin may be limited by the availability of ornithine. The activities of ornithine carbamoyltransferase (EC 2.1.3.3) and ornithine δ-aminotransferase (EC 2.6.1.13) are negatively correlated with the antibiotic-synthesizing ability of u.v. mutants of *Bacillus subtilis*. Besides, high productive mutants of two strains of *B. subtilis* were found to be deficient in ornithine δ-aminotransferase. Induction of arginine metabolism in the stationary phase of growth of *B. subtilis* has been referred to successive stages of sporulation, and the size of ornithine pool to the proteolytic activities of the investigated mutants.

MATERIALS AND METHODS

Organism and culture conditions. The bacterial strains used in this study were all derivatives of two *B. subtilis* isolates: *152* and *A*, obtained at this Institute. The initial isolates and u.v. mutants of strain *A* differed in the ability to synthesize bacitracin and extracellular protease. The antibiotic yield and proteolytic activity in the 40 h cultures of strain *152*, strain *A* and its eight u.v. mutants are presented in Table 1.

Table 1

Production of bacitracin and extracellular protease in the 40 h cultures of B. subtilis *strains*

Strain	Bacitracin (units/ml)	Protease (units/ml)
152	55	4150
A	90	4170
u.v. mutants of *A*:		
p38	78	1350
sp51	78	1030
p6	78	1000
rp77	61	150
rp82	55	550
rp79	48	200
rp49	17	1700
A/66	0.5	170

Microorganisms were grown in 500 ml Erlenmayer flasks on rotary shaker (240 rot./min) at 32°C for 40 h on the soluble complex medium containing: soya flour extract, 5%; starch, 2%; $CaCO_3$, 1%; $MgSO_4$, 0.05%; $MnCl_2$, 0.0002%; $(NH_4)_2SO_4$, 0.2%; pH 6.45.

Determination of growth and sporulation. The number of colony forming units (c.f.u.) and that of the heat-resistant spores were estimated by the plate method, the latter after heating the culture at 75°C for 30 min.

Determination of bacitracin. Bacitracin was determined according to Grove & Randall (1955) by the cylinder-plate microbiological method using *Micrococcus flavus* ATCC 10240 as a test organism. The bacitracin unit defined in W.H.O. Technical Report Series no. 384 (Genève, 1968) corresponds to 30 μg of bacitracin.

Preparation of extracts. Each 8 h the contents of 3 fermentation flasks were pooled, the bacterial cells were spun down at 12 000 rev./min for 30 min at 0°C, and washed twice with cold water. The cells were suspended in 2 vol. of 0.05 M-Tris buffer, pH 7.0, sonicated in M.S.E. sonicator at 20 Kc for 10 min, centrifuged, and the obtained supernatant was used for the enzyme assay.

To extract the metabolites, 2 vol. of 10% (w/v) perchloric acid was added to the washed cells and homogenized in a glass homogenizer. After centrifugation, the

deproteinized supernatant was neutralized with 20% KOH and separated from potassium perchlorate, which precipitated on 30 min standing in ice.

Enzyme assay. Arginase (EC 3.5.3.1) was determined according to Greenberg (1955). The incubation mixture contained: L-arginine-HCl, 5 μmoles; $MnCl_2$, 0.05 μmole, and extract (about 5 mg protein) in 1 ml of 0.25 M-glycine-NaOH buffer, pH 9.5. After 10 min incubation at 30°C the reaction was stopped by adding 1 ml of 6 N-phosphoric acid and ornithine formed was measured after Chinard (1952).

Ornithine δ-aminotransferase (EC 2.6.1.13) was assayed according to DeHauwer *et al.* (1964). The incubation mixture contained: ornithine-HCl, 20 μmoles; α-ketoglutarate, 20 μmoles; pyridoxal phosphate, 50 μg; *o*-aminobenzaldehyde, 0.2 mg, and extract (about 15 mg protein) in 3 ml of 0.05 M-triethylamine buffer, pH 8.5. After 30 min incubation at 30°C the reaction was stopped by adding 1 ml of 10% trichloroacetic acid and the extinction of the formed coloured complex was measured at 430 nm; a unit has been defined as that amount of the enzyme, which produces formation of the product resulting in $\Delta E_{430\,nm}=0.001$.

Ornithine carbamoyltransferase (EC 2.1.3.3) was determined after Jones *et al.* (1962). The incubation mixture contained: carbamoylphosphate lithium salt, 50 μmoles; L-ornithine-HCl, 50 μmoles, and extract (about 5 mg protein) in 0.5 ml of 5 mM-Tris buffer, pH 8.5. After 15 min incubation at 30°C, 1 ml of 5% trichloroacetic acid was added and citrulline was determined according to Archibald (1944).

Alkaline phosphatase (EC 3.1.3.1) was determined after King & Wootom (1956), glucose-6-P-dehydrogenase (EC 1.1.1.49) and isocitrate dehydrogenase (EC 1.1.1.42) after Kornberg & Horecker (1955) and Kornberg (1955), respectively, at 30°C.

Extracellular protease was estimated in the cell-free supernatant obtained on centrifugation at 12 000 rev./min for 30 min at 0°C. The incubation mixture contained: 2.9 ml of 1% casein in 3.0 ml of 0.4-glycine-NaOH buffer, pH 11, and 0.1 ml of supernatant suitably diluted (100 - 1000 units). After 40 min incubation at 40°C, 3 ml of 10% $HClO_4$ was added and the extinction measured at 280 nm in the deproteinized solution. One enzymic unit was defined as that amount of the enzyme which produces $\Delta E_{280\,nm}=0.001$, equivalent to 3.2 nmoles of tyrosine.

Determination of amino acids. The amino acid fraction was separated on Amberlite IR-120 (H^+) from the neutralized perchlorate extracts of the cells and media. The pool of free amino acids was estimated by the Rosen ninhydrin method as modified by Alberti & Bartley (1963). The amount of bound amino acids was determined by the same method after hydrolysis in 6 M-HCl at 120°C for 18 h.

Arginine was assayed enzymically after Greenberg (1955), with arginase prepared from rat liver according to Schimke (1964); the preparation contained 250 units/ml. Ornithine, formed in the reaction, was estimated according to Chinard (1952).

Proline was separated from ornithine on permutit (50 mg/μmol) and estimated by the method of Wren & Wiggal (1965). Ornithine adsorbed on permutit was eluted with 1 M-NaOH (2×1 ml) and determined subsequently by the same method.

Citrulline was assayed according to Archibald (1944) and glutamate enzymically with glutamate dehydrogenase after Bernt & Bergmeyer (1962).

The results of amino acid assays were expressed in μmoles per gram wet weight obtained on 30 min centrifugation of bacterial cultures at 12 000 rev./min.

Determination of protein. Protein was estimated according to Lowry (Layne, 1957).

Materials. Glutamate dehydrogenase (20 mg/ml), glucose-6-phosphate disodium salt, carbamoylphosphate lithium salt and NAD^+ were products of C.F. Boehringer (Mannheim, G.F.R.). Amino acids: L-ornithine-HCl, L-arginine and pyridoxal phosphate were from Calbiochem (Lucerne, Switzerland). Tris was a product of A. G. Fluka (Buchs, Switzerland), and ninhydrin of VEB Laborchemie APOLDA (Berlin). Permutit Q was from Permutit (New York, U.S.A.), casein (soluble) from B.D.H. (Poole, Dorset, England). Other reagents were from Fabryka Odczynników Chemicznych (Gliwice, Poland).

RESULTS

Arginase, ornithine δ-aminotransferase and ornithine carbamoyltransferase in biosynthesis of bacitracin

The affinity of ornithine carbamoyltransferase for ornithine, expressed in K_m values (4.2×10^{-6} M) was by about two orders of magnitude higher than the affinity of both catabolic enzymes: ornithine δ-aminotransferase and arginase towards their substrates (K_m 3.3×10^{-4} M and 6.6×10^{-4} M, respectively).

To examine the relation of arginine metabolism to biosynthesis of bacitracin we have referred the activity of these three enzymes to sporogenesis since in *B. subtilis*

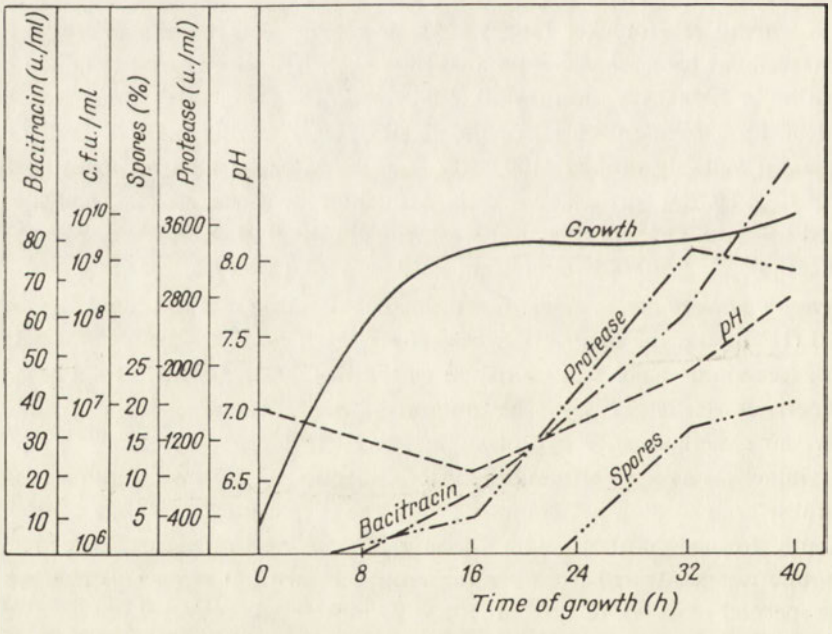

Fig. 1. Growth, sporulation, protease activity and bacitracin formation in *B. subtilis*, strain *A*. Bacitracin assayed with *Micrococcus flavus* ATCC 10240, and protease following 40 min incubation at 30°C in 0.4 M-glycine buffer at pH 11.0. Definition of units as described in Methods.

formation of peptides showing antibiotic activity is intimately connected with this development stage (Schaeffer, 1967; Mandelstam & Waites, 1968). The period at which arginase, ornithine carbamoyltransferase and ornithine δ-aminotransferase were induced was designated by means of enzymic markers correlated by Warren (1968) with the successive morphological stages of sporulation in *B. subtilis*.

Traces of extracellular protease and bacitracin, characteristic for initiation of sporogenesis, were observed in *B. subtilis* under our experimental conditions prior to 8 h of growth (Fig. 1). Synthesis of both these extracellular products extended to the late stages of sporulation, as indicated by the release of the heat-resistant spores. Induction of arginase (tenfold) and ornithine carbamoyltransferase (sevenfold) took place at the early stages of sporulation, i.e. during the forespore formation, following the induction of extracellular protease and alkaline phosphatase. Degradation of ornithine by ornithine δ-aminotransferase was induced (tenfold) distinctly later as indicated by the activity of glucose-6-phosphate dehydrogenase and the appearance of heat-resistance of developing spores. The induction of this enzyme took place therefore at stage V during development of spore coat.

The results of induction studies presented in Fig. 2 refer to mutant *A/66* producing

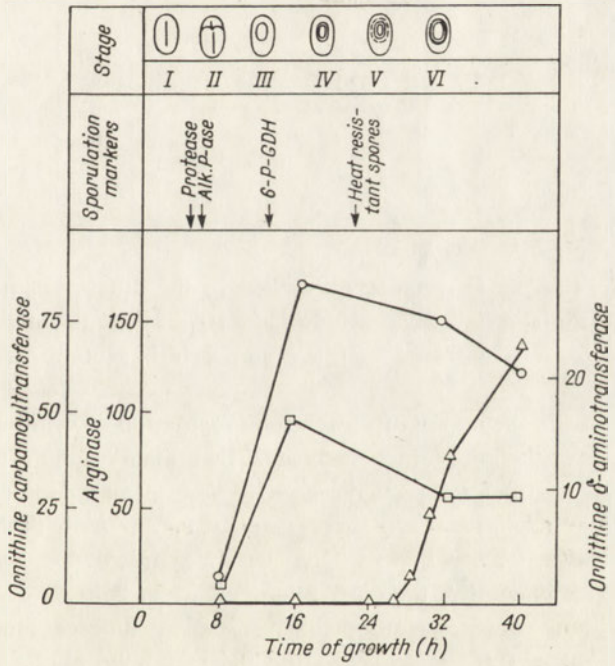

Fig. 2. Induction of arginine metabolism during growth of *B. subtilis*, mutant *A/66*. The activities of ornithine carbamoyltransferase (□) and arginase (○) expressed in nmol/mg protein/min, and that of ornithine δ-aminotransferase (△) in units/mg protein/min. Data concern 3 experiments, each involving 3 separate cultures. Successive stages of sporulation were marked by estimating induction of protease, alkaline phosphatase (Alk. P-ase), 6-P-glucose dehydrogenase (6-P-GDH) and the appearance of heat-resistant spores according to Warren (1968). The assay procedures described in Methods.

low yield of bacitracin since it has been found that strain *152* and strain *A* which synthesize high amounts of antibiotic are deficient in ornithine δ-aminotransferase. This indicated that genetic block in degradation of ornithine by ornithine δ-aminotransferase could enhance bacitracin formation by sparing ornithine pool for the synthesis of antibiotic. To check this supposition ornithine was added in the post-logarithmic phase of growth to the 16 h cultures of strain *A* deficient in ornithine δ-aminotransferase and to the cultures of mutant *rp49* showing high activity of this enzyme. It was found that in the latter cultures the addition of ornithine resulted in an about twofold stimulation of bacitracin formation, but was ineffective in the case of high productive strain *A* blocked in the ornithine δ-transamination step (Table 2).

Table 2

Effect of ornithine on bacitracin formation in B. subtilis

In the 40 h cultures, bacitracin was determined in the medium and ornithine in the extracts of sonicated cells, as described in Methods. Mean values from 3 separate experiments are given.

Organism	Ornithine added (mM)	Bacitracin		Ornithine (μmol/g wet wt.)
		units/ml	%	
Strain *A* (high-productive)	—	77	100	0.090
	30	64	83	0.540
	50	76	99	0.520
Mutant *rp49*	—	22	100	0.068
(low-productive)	10	28	127	0.088
	30	42	190	0.120

This different response cannot be ascribed to the differences in permeability of ornithine since the addition of ornithine (30 mM) resulted in only twofold increase in the intracellular concentration of this amino acid in mutant *rp49* and fivefold increase in strain *A*.

The role of ornithine δ-aminotransferase in regulation of ornithine pool available for the synthesis of the antibiotic is confirmed by a highly significant negative correlation coefficient (−0.78) between the activity of ornithine δ-aminotransferase and antibiotic yield in *B. subtilis* strain *A* and its eight u.v. mutants (Table 3). The correlation between the activity of ornithine carbamoyltransferase and the antibiotic formation was also negative, the correlation coefficient being −0.92. This indicates contribution of both enzymes, metabolizing ornithine in different metabolic pathways, in the withdrawal of this amino acid from the metabolic pool used for antibiotic synthesis. It should be noted that the activity of arginase was not correlated with antibiotic-synthesizing ability of the investigated mutants (corr. coeff. −0.02).

The deficiency of ornithine δ-aminotransferase in strain *A* producing high yield of bacitracin resulted in lower accumulation of glutamate and proline in the cells as compared with *A/66* synthesizing a trace amount of the antibiotic (Fig. 3). The importance of catabolic pathway of arginine metabolism in the synthesis of proline

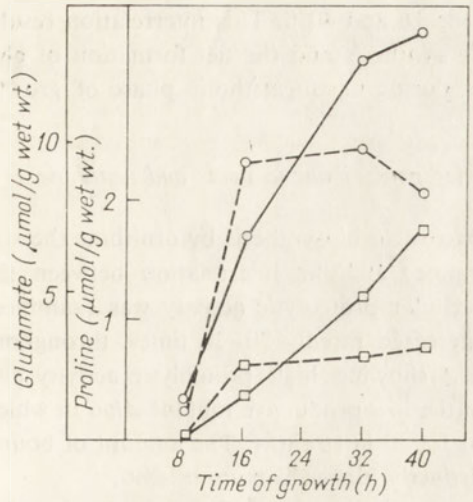

Fig. 3

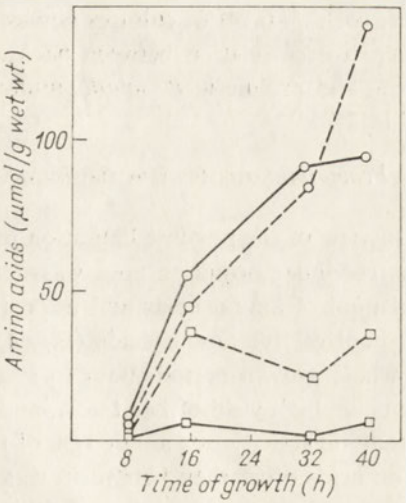

Fig. 4

Fig. 3. Intracellular concentration of glutamate and proline in *B. subtilis* mutants: strain *A* (solid line) and mutant *A/66* (broken line). Glutamate (○) determined enzymically and proline (□) colorimetrically with ninhydrin following adsorption of ornithine on permutit in the perchlorate extract of the cells. The results are means of 4 experiments involving 3 separate cultures.

Fig. 4. The pool of free (○) and bound (□) amino acids in *B. subtilis* strain *A* (solid line) and mutant *A/66* (broken line). The amino acids were separated on Amberlite IR-120 and estimated by the ninhydrin method prior to and after acid hydrolysis (see Methods). The results are means of 4 experiments involving 3 separate cultures.

Table 3

Correlation between bacitracin formation and arginine metabolism in strain A *and its eight u.v. mutants*

Correlation coefficients between antibiotic yield and enzymic activities refer to 40 h cultures. In calculation of correlation coefficients between yield and amino acid concentration, the increase of the respective pools between 16 and 40 h of growth, paralleling bacitracin formation, was taken into account. Enzymic activities, amino acids and bacitracin were determined as described in Methods.

Antibiotic : enzymes or amino acids	Correlation coefficient
Bacitracin : arginase	−0.02
Bacitracin : ornithine δ-aminotransferase	−0.78
Bacitracin : ornithine carbamoyltransferase	−0.92
Bacitracin : ornithine	−0.27
Bacitracin : glutamate	−0.90
Bacitracin : proline	−0.76

and glutamate in *B. subtilis* mutants is confirmed by a high positive correlation coefficient between the activity of ornithine δ-aminotransferase in the 40 h cultures and the increase in concentration of glutamate (corr. coeff. +0.85) and proline

(corr. coeff. +0.74) in cultures between the 16 and 40 h. This interrelation results in negative correlation between bacitracin synthesis and the net formation of glutamate and proline in *B. subtilis* mutants, during postlogarithmic phase of growth (Table 3).

Proteolytic activity and the intracellular pool of amino acids and ornithine

In view of the possible limitation of bacitracin biosynthesis by ornithine the size of intracellular ornithine pool was determined and the interrelation between the total pool of amino acids and the extracellular proteolytic activity was examined. The pool of free amino acids (Fig. 4) increased rapidly 20 - 30 times, throughout the whole growth period, both in strain *A* showing high proteolytic activity and producing high yield of bacitracin, and in the low productive mutant *A/66* in which proteolytic activity was about 1/30 of that found in strain *A*. The amount of bound amino acids other than bacitracin was distinctly higher in mutant *A/66*.

Concentration of arginine, ornithine and citrulline, similarly as the total amino acid pool, increased in the logarithmic and postlogarithmic phase of growth (Fig. 5). Concentration of citrulline and ornithine in strain *A* and mutant *A/66* was practically the same up to 32 h of growth. The differences were only observed in the late 40 h cultures in which concentration of citrulline in mutant *A/66* was twice as high as, and that of ornithine half that, in strain *A*.

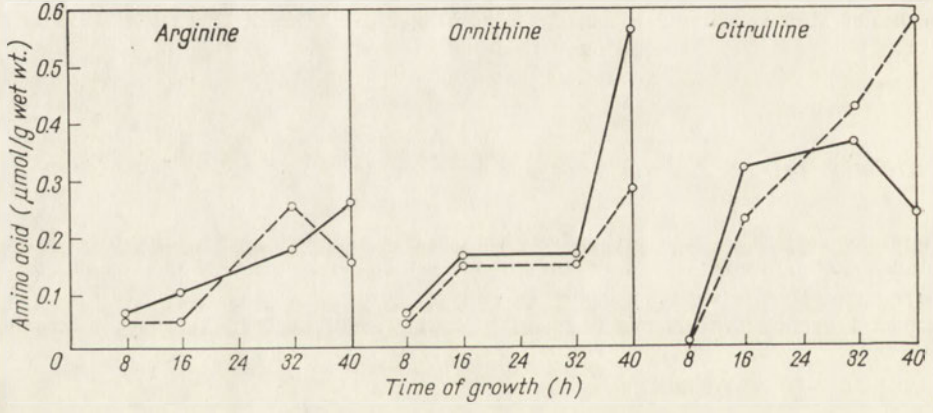

Fig. 5. Intracellular concentration of arginine, ornithine and citrulline in *B. subtilis* strain *A* (solid line) and mutant *A/66* (broken line). The results are means of 4 experiments involving 3 separate cultures.

It was found that the size of total pool of free amino acids and that of ornithine measured in eight u.v. mutants was neither correlated with proteolytic activity nor with bacitracin-synthesizing ability (Table 4).

This lack of relation between proteolytic activity and the bacitracin formation was confirmed in the experiments with strain *A* when proteolytic activity was stimulated by glyoxylate or inhibited by acetate (Table 5); both these compounds were found to be the effective agents changing proteolytic activity of *B. subtilis*. Acetate and

Table 4

Correlation between proteolytic activity, bacitracin synthesis and amino acid pool in strain A *and its eight u.v. mutants*

Correlation coefficients between protease activity and bacitracin yield refer to 40 h cultures. Calculation of coefficients between activity of protease and concentration of amino acids as in legend to Table 3. Determinations of protease, bacitracin and amino acids were performed as described in Methods.

Protease : bacitracin or amino acids	Correlation coefficient
Protease : bacitracin	+0.47
Protease : amino acid pool	+0.28
Protease : ornithine	−0.046

Table 5

Effect of glyoxylate and acetate on the intracellular amino acid pool and proteolytic activity in B. subtilis *strain* A

Glyoxylate or acetate were added to 16 h cultures. Bacitracin yield, protease activity and amino acids were determined in 40 h cultures as described in Methods. Mean values of 3 separate experiments are given.

Addition	Bacitracin (units/ml)	Protease (units/ml)	Amino acid pool (μmol/g wet wt.)					
			Total		Arg	Orn	Citr	Pro
			free	bound				
None	80	2900	64	17	0.26	0.18	0.37	0.46
Glyoxylate								
25 mM	15	5870	72	28	0.30	0.16	0.30	0.54
50 mM	4	8250	72	30	0.17	0.18	0.40	0.61
Acetate								
100 mM	72	150	105	0				

Table 6

Effect of glyoxylate on arginine metabolism and isocitrate dehydrogenase activity in B. subtilis *strain* A

Glyoxylate and ornithine were added in concentration of 50 mM to 16 h cultures. Bacitracin yield, arginase and isocitrate dehydrogenase activities and amino acid concentration were determined in 40 h cultures. Mean values of 3 separate experiments are given.

Addition	Bacitracin (%)	Activity (μmol/mg protein/min)		Concentration (μmol/g wet wt.)	
		Arginase	Isocitrate dehydrogenase	Arg	Orn
None	100	0.10	0.024	0.20	0.09
Glyoxylate	2	0.04	0.003	0.35	0.07
Ornithine	110	0.35	0.021	0	0.52
Glyoxylate+ ornithine	2	0.13	0.004	0.20	0.50

glyoxylate were added at the early phase of postlogarithmic growth. Data presented in Table 5 show that an about threefold increase in proteolytic activity caused by glyoxylate had no effect on the intracellular pool of free amino acids; the pool of bound amino acids was slightly increased. An about twentyfold decrease in proteolytic activity caused by acetate was associated with a slight increase in the size of free amino acid pool and a decrease in concentration of bound amino acids to non-detectable amounts. The changes in proteolytic activity were not reflected in concentration of arginine, citrulline or proline. Acetate did not affect antibiotic yield, while glyoxylate decreased drastically bacitracin formation. Moreover, it has been demonstrated (Table 6) that glyoxylate diminished the activity of arginase and isocitrate dehydrogenase, but restoration of arginase activity and the increase of intracellular ornithine pool by ornithine (50 mM) added simultaneously with glyoxylate in equimolar concentration, did not abolish the glyoxylate effect on bacitracin formation. Inhibition of antibiotic synthesis by glyoxylate is thus independent from its effect on proteolytic activity.

DISCUSSION

The presented results indicate that biosyntheis of bacitracin may be limited by the availability of ornithine. This limitation, however, was observed only with the low productive u.v. mutant showing high activity of ornithine δ-aminotransferase. The addition of 30 mM-ornithine to the cultures of the mutant doubled biosynthesis of bacitracin, whereas even 50 mM-ornithine was without effect on the yield of the high productive strain *A* with the genetic block in ornithine δ-aminotransferase.

The role of ornithine δ-aminotransferase in regulation of the antibiotic synthesis was confirmed by a highly significant negative correlation between the activity of this enzyme and bacitracin yield in eight u.v. mutants of *B. subtilis*. Moreover, a similar high negative correlation has been found between the yield of bacitracin and the activity of ornithine carbamoyltransferase. This suggests that both anabolic and catabolic routes of arginine metabolism deplete ornithine pool used for bacitracin formation. In connection with these results it should be noted that the total intracellular ornithine pool is not related to the bacitracin-synthesizing ability (corr. coeff. −0.27). This might indicate compartmentation of the ornithine pool, as it was postulated for arginine by Davis (1968).

Diaminobutyric acid, another non-protein amino acid, has been found to be a limiting factor in biosynthesis of colistin. The addition of this amino acid to the cultures of *B. colistinus* Koyama at concentration of 80 mM doubled colistin synthesis (Ito *et al.*, 1969; 1970). However, as no data are available on regulation of the synthesis and degradation of this constituent of colistin, no further comparison can be made with our results.

The interrelationship between arginine metabolism and bacitracin formation is also confirmed by the induction in *B. subtilis* of all three investigated enzymes of arginine metabolism in the postlogarithmic phase of growth during which bacitracin is formed. It is of interest that induction of arginase, the catabolic enzyme,

and carbamoyltransferase, the anabolic enzyme, are coincident and precede induction of ornithine δ-aminotransferase, which takes place in the later stages of sporulation during the appearance of heat-resistant spores. The mechanism of this delay in the induction of ornithine δ-aminotransferase has not been elucidated. The common genetic control of anabolic and catabolic enzymes of arginine metabolism has been postulated by Wiame (1970) in yeasts and by Cybis *et al.* (1972) in *Aspergillus nidulans*. A different regulatory system of arginine metabolism has been found in *B. licheniformis*. The arginine catabolic pathway in this organism is induced during sporogenesis (Laishley & Bernlohr, 1968). The three "sporulation enzymes": arginase, ornithine δ-aminotransferase and Δ-pyrroline-5-carboxylate dehydrogenase, are derepressed by arginine and are under catabolite repression control. In contrast to catabolic pathway, the maximum activity of ornithine carbamoyltransferase is observed in the vegetative cells. During sporulation this enzymic activity is only about half that found in the vegetative cells. Arginine was found to be a repressor of carbamoyltransferase (Ramaley & Bernlohr, 1966). The differences found in the control of arginine metabolism in two sporulating bacilli, *B. subtilis* and *B. licheniformis*, are worth mentioning.

The correlation between arginine metabolism and bacitracin yield in *B. subtilis* is not affected by the extracellular proteolytic activity. The intracellular pool of free amino acids in the investigated strains differing in proteolytic activity is not significantly differentiated. The induction of this activity by glyoxylate or repression by acetate had no effect on the amino acid pool. These results are at variance with the suggestion that production of protease is related to sporulation and protein turnover (Mandelstam & Waites, 1968; Schaeffer, 1969; Hageman & Carlton, 1973) and confirm the conclusion of Slapikoff *et al.* (1971) that sporulation of *B. brevis* is accomplished without production of detectable amounts of extracellular protease and does not require extensive protein turnover. It seems that more and more evidence is accumulating pointing to the lack of relation between sporulation and antibiotic synthesis (Haavik & Thommassen, 1973).

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ANABOLICZNA I KATABOLICZNA PRZEMIANA ARGININY U *B. SUBTILIS* SYNTETYZUJĄCEGO BACYTRACYNĘ

Streszczenie

1. Arginaza i karbamoilotransferaza ornitynowa są indukowane we wczesnym stadium sporogenezy, a transaminaza ornitynowa podczas tworzenia okrywy spor.

2. Aktywność enzymów metabolizujących ornitynę w anabolicznej i katabolicznej przemianie argininy są ujemnie skorelowane z syntezą bacytracyny, podczas gdy synteza antybiotyku nie zależy od czynności arginazy.

3. Ornityna ogranicza syntezę bacytracyny u nisko produkcyjnego mutanta wykazującego wysoką czynność transaminazy; wpływu tego nie obserwowano u szczepów wysokowydajnych z blokiem w transaminazie δ-ornitynowej.

4. Wielkość puli wewnątrzkomórkowych aminokwasów nie zależy od czynności pozakomórkowej proteazy u mutantów ani od wpływu czynników zwiększających (glioksalan) lub hamujących (octan) czynność proteolityczną.

Received 21 December, 1973.

ACTA BIOCHIMICA POLONICA
Vol. 21 1974 No. 2

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PURIFICATION AND PARTIAL CHARACTERIZATION OF PROTEASE FROM CALF THYMUS CHROMATIN *

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The neutral protease from calf thymus chromatin was purified 1800-fold, and was found to be homogeneous on acrylamide-gel electrophoresis. pH optimum for the enzyme is about 8.5 with total histone as substrate. The enzyme hydrolyses preferentially histones (all five fractions at a similar rate) and basic ribosomal proteins. It has no trypsin or chymotrypsin-like esterase activity and does not degrade β-naphthylamides of alanine, glycine, arginine, lysine and histidine. Activity of the enzyme is not inhibited by chelating or thiol-blocking agents, nor by sodium bisulphite. The enzyme has a molecular weight 15 400 ± 1000.

Furlan & Jericijo (1967a,b) have described some of the properties of a neutral protease found in calf thymus nuclei. They suggested that the enzyme occurs exclusively in nucleus as a component of nucleoprotein complex. A similar and most probably the same enzyme has been found by Bartley & Chalkley (1970) in nucleohistone preparations from cells of calf thymus, lung and intestine and from mouse Ehrlich ascites tumour. The presence of a neutral protease has been also demonstrated in chromatin preparations from calf thymus (Kurecki *et al.*, 1971), hog lung, spleen and kidney (Kurecki *et al.*, 1972), and rat liver (Garrels *et al.*, 1972). A characteristic feature of the chromatin enzyme is its ability to degrade only two (*f1* and *f3*) out of the five histone fractions when they are part of nucleohistone or chromatin complex (Furlan *et al.*, 1968; Bartley & Chalkley, 1970; Kurecki *et al.*, 1971).

A neutral protease has been isolated from rat liver chromatin and purified 20 - 30 fold by Garrels *et al.* (1972). The procedure of isolation and partial purification (about 50-fold) of neutral protease from calf thymus chromatin has been also described by us (Kurecki & Toczko, 1972).

In the present paper the procedure of isolation of highly purified (about 1800--fold) enzyme preparation from calf thymus chromatin is described and some data on its catalytic and molecular properties are given.

* This work was partly supported by the Committee of Biochemistry and Biophysics, Polish Academy of Sciences.

MATERIALS AND METHODS

Material. Calf thymus was isolated immediately after slaughtering of the animals, frozen in solid CO_2 and stored at $-20°C$ for not longer than 2 weeks.

Crude enzyme preparation. Calf thymus (300 g) free of connective tissue was cut into small pieces and homogenized in a mixer for 2 min with 1000 ml of homogenization solution (0.075 M-NaCl in 0.024 M-EDTA, pH 8.0). The homogenate was filtered through 4 layers of cheese-cloth and centrifuged at 1500 *g* for 15 min, the sediment suspended in 1000 ml of homogenization solution and centrifuged as above. The obtained sediment was resuspended and washed three times with 1000 ml portions of 0.05 M-Tris-HCl buffer, pH 8.0, and centrifuged each time at 1500 *g* for 15 min. After the last centrifugation, the crude chromatin preparation was suspended in 500 ml of 0.01 M-Tris-HCl buffer, pH 8.0, to which 1.5 M-NaCl solution was added dropwise, with continuous stirring, to the final concentration of 0.15 M. The suspension was stirred for another 30 min, centrifuged, and the sediment was collected.

For separation of the dissociated protease and histones from the remaining nucleoprotein complex, extraction with cold HCl was used, which had been found by Kurecki *et al.* (1971) to be more convenient than NaCl extraction. To the above sediment, 300 ml of cold 0.25 M-HCl was added dropwise during 4 - 5 min with continuous and vigorous stirring and the suspension was centrifuged at 2000 *g* for 10 min. The clear supernatant was immediately adjusted to pH 4.5 - 4.7 with 2 M-NaOH. To this solution 4 volumes of acetone cooled to $-20°C$ were slowly added, with continuous stirring, and after 30 min in a cold-room centrifuged at 1000 *g* for 10 min. The precipitated protein was suspended in 200 ml of cold acetone, filtered on Buchner funnel, washed with two 100 ml portions of acetone and dried, first in a stream of air and then *in vacuo* over KOH.

Chromatography on Sephadex G-75. The crude enzyme preparation, 2 g, was dissolved in 30 ml of 0.5 M-NaCl in 0.01 M-acetate buffer, pH 4.2 (buffered salt solution) and centrifuged at 3000 *g* for 10 min. The clear supernatant was applied on Sephadex G-75 column (50 × 4.5 cm, V_t 700 ml) equilibrated with the buffered salt solution, and the elution was conducted with the same solution at a rate of 50 - 60 ml/h. The eluate was collected in 10-ml fractions and assayed for protein content and enzyme activity. Fractions containing the protease (eluted at 0.52 - 0.68 V_e/V_t) were pooled and mixed with 4 volumes of cold acetone ($-20°C$), left for 1 hour in a cold-room and then centrifuged at 2000 *g* for 10 min. The sediment was washed with a small volume of acetone, centrifuged as above and dried *in vacuo* over KOH.

Autolysis. The obtained preparation, 500 mg of protein, was dissolved in 15 ml of 0.1 M-Tris-HCl buffer, pH 8.0, and dialysed for 9 h against 2 litres of 0.025 M-Tris-HCl buffer, pH 8.0, at 37°C, then centrifuged at 5000 *g* for 10 min, and the clear supernatant was fractionated on a Sephadex G-75 column (65 × 2.0 cm, V_t 190 ml). The pooled active fractions were further processed as described above.

Preparative electrophoresis. Electrophoresis was conducted in the Porath apparatus (LKB, Sweden). The protease preparation, 5.0 ml, containing 2.5 - 5.0 mg/ml

of protein in 0.1 M-acetate buffer, pH 4.0, was applied to the column filled with Sephadex G-50 (200 ml), then the column was washed with 10 ml of 0.1 M-acetate buffer, pH 4.0. Electrode vessels were filled with the same buffer and electrophoresis was run for 12 h at 500 V (30 - 40 mA). After electrophoresis the protein was eluted from the column with 0.1 M-acetate buffer, pH 4.0, in 3-ml fractions at a rate of 25 ml/h. The fractions containing the enzyme were pooled, dialysed overnight against deionized water and lyophilized. The obtained preparation was used for determination of molecular weight and catalytic properties.

Determination of enzyme activity. To 0.5 ml of enzyme solution, 0.4 ml of 0.5% histone (or another protein) in 0.1 M-Tris-HCl buffer, pH 8.0, and 0.1 ml of Tris-HCl buffer, pH 8.0, were added and incubation was carried out at 37°C. After 6 h 2 ml of 20% trichloroacetic acid (TCA) was added and the mixture centrifuged at 3000 *g* for 10 min. In control assays, the components of incubation mixture were added to 2 ml of 20% TCA. In 1 ml of the supernatant, the protein degradation products were determined by the method of Lowry *et al.* (1951) except that for neutralization of the TCA present in the sample, 2% $NaCO_3$ in 0.3 M-instead of in 0.1 M-NaOH, was used.

For activity measurements, the enzyme solution was diluted to such an extent that the formed products gave an extinction at 660 nm exceeding that of control by 0.150 - 0.250. One enzyme unit was defined as that amount of enzyme protein which gave an amount of peptides equivalent to 1 μmol of tyrosine.

Activity toward β-naphthylamide derivatives of amino acids was determined colorimetrically as described by Goldbarg & Rutenburg (1958). Incubation was carried out for 1 h at 37°C with 0.4 mg/ml of substrate in 0.1 M-Tris-HCl buffer, pH 8.0. Activity toward methyl ester of *p*-toluenesulphonyl-L-arginine (TAME) was determined by the method of Hummel (1959) and toward ethyl ester of benzoyl-L-tyrosine (BTEE) by the method of Laskowski (1955). Incubation with 0.01 M-substrates was performed in 0.05 M-Tris-HCl buffer, pH 8.0, for 30 min at 37°C.

Effect of activators and inhibitors. The enzyme solution in 0.1 M-Tris-HCl buffer, pH 8.0, was preincubated for 10 min at 37°C with the appropriate compound, then the activity was determined using total histone as substrate. In the control sample, the enzyme solution was preincubated with 0.1 M-Tris-HCl buffer, pH 8.0.

Polyacrylamide-gel electrophoresis. This was performed by the method of Bonner *et al.* (1968) and of Johns (1967). To each gel 20 - 100 μg of protein were applied in a volume up to 100 μl. Gels were stained with 0.1% Amido Black in 7% acetic acid, destained by exhaustive washing with 7% acetic acid, then scanned using an ERJ 65 densitograph (Carl Zeiss, Jena, G.D.R.).

DNA determination. DNA in chromatin was determined according to Munro & Fleck (1966) assuming that one E_{260} unit is equivalent to 44.3 μg of DNA.

Protein determination was made by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Molecular weight determination. Approximate molecular weight was estimated by gel filtration on Sephadex G-75 column (40 × 1.5 cm). V_0 was estimated using

Dextran Blue 2000 and the relation between K_{av} and molecular weight, using lysozyme and histone *f1* as standards. The column was equilibrated with 0.5 M-NaCl in 0.01 M-acetate buffer, pH 4.2, and eluted at a rate of about 25 ml/h.

Reagents. Sephadex G-75 was obtained from Pharmacia (Uppsala, Sweden); acrylamide, *N*,*N*-bisacrylamide, and TEMED were from Serva (Heidelberg, G.F.R.); synthetic substrates (TAME and BTEE) and pancreatic ribonuclease were from Koch-Light Lab. (London, England), Tris from Fluka AG (Buchs SG, Switzerland), bovine serum albumin from Michrome (London, England), haemoglobin and casein from Merck (Darmstadt, G.F.R.), and egg white lysozyme from Reanal (Budapest, Hungary). Total histones were prepared from calf thymus according to Bonner *et al.* (1968). Histone *f1* was isolated from calf thymus by the method of Johns (1964). Basic ribosomal protein was prepared from rat liver as described by Staroń *et al.* (1971). Other reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

RESULTS

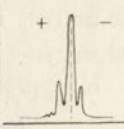
The results of purification of chromatin protease at particular stages are summarized in Table 1.

The crude HCl-extract from chromatin contained 12 - 15 mg/ml of protein with specific activity of 0.028 unit per mg. Analysis of activity in pH range 2.0 - 10.0 showed practically no contamination by acid protease(s).

Table 1

Purification of protease from calf thymus chromatin

Data of purification procedure were calculated per 1 kg of fresh calf thymus. The polyacrylamide-gel electrophoretic patterns obtained at the successive stages of purification, are also presented.

Purification step	Protein (mg)	Activity		Purification	Yield (%)	Densitometer scans
		total (units)	specific (units/mg)			
Crude enzyme preparation	20 000	560	0.028	1.0	100	
Sephadex G-75 chromatography (fractions 0.52 - 0.68 V_e/V_t)	900	440	0.49	18	78	
Autolysis and Sephadex G-75 chromatography (fractions 0.42 - 0.62 V_e/V_t)	9.8	293	29.9	1092	52	
Preparative electrophoresis	2.4	122	50.8	1814	21	

It has been shown previously (Kurecki & Toczko, 1972) that Sephadex G-75 gel filtration of the crude enzyme extract removed about 95% of inactive protein, the degree of purification at this step being about 20-fold.

A further, very efficient step, which gave an about 50-fold purification, consisted in autolysis at 37°C and pH 8.0 for 9 h, with simultaneous dialysis, and separation of autolysis products on Sephadex G-75 column (Fig. 1). The molecular weight of the bulk of the products formed was smaller than that of the enzyme, which made possible easy separation of the enzyme.

The obtained enzyme preparation resolved on electrophoresis into two protein peaks, the protease activity being located in the peak showing higher mobility (Fig. 2). After this last step, a 1800-fold purification was achieved as compared with the crude enzyme, with the overall yield of enzyme of 20%.

Polyacrylamide-gel electrophoresis patterns at successive stages of protease purification are included in Table 1. The densitogram of the final preparation shows the intensive and relatively broad band of smaller mobility than that of the slowest migrating histone *f1*. Considering the degree of purification of enzyme it can be assumed that this band represents the protease. It was not visible at the first two stages of purification when histones still formed the dominating bands.

Properties of chromatin protease

For studying the properties of the enzyme, the purified preparation was used.

The effect of pH on protease activity was measured with total histone from calf thymus as substrate (Fig. 3). The enzyme was most active at about pH 8.5. The

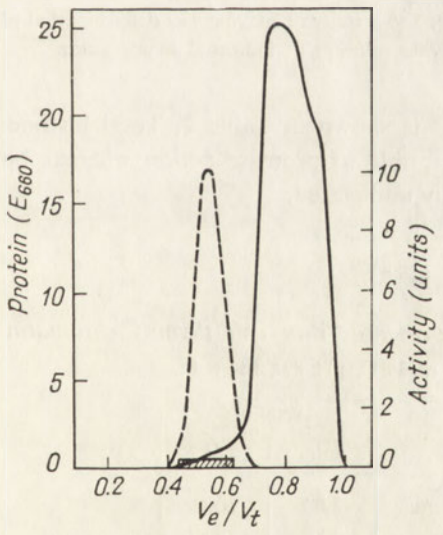


Fig. 1

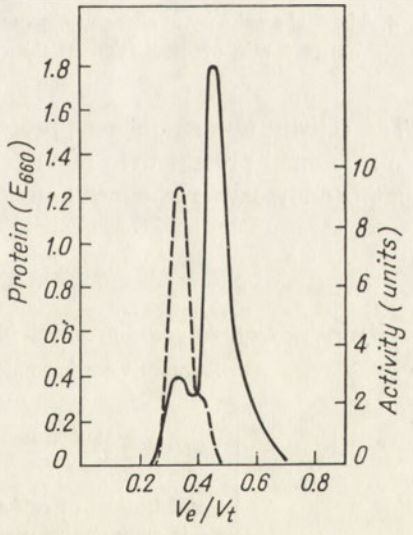


Fig. 2

Fig. 1. Gel chromatography on Sephadex G-75 of the enzyme preparation after 9-h autolysis. ——, E_{660}; - - - -, activity at pH 8.0 with total histone as substrate.

Fig. 2. Distribution of proteins and enzyme activity after preparative electrophoresis. ——, E_{660}; - - - -, activity at pH 8.0 with total histone as substrate.

decrease of activity at acid pH (to pH 3.5) was found to be reversible whereas the decrease at pH above 9.0 was due to irreversible inactivation of the enzyme.

Temperature dependence of protease activity was measured after 5 min of preincubation of the enzyme at temperatures ranging from 37° to 80°C. Up to 50°C the activity was unchanged; higher temperatures resulted in partial inactivation, and 80°C caused a complete loss of enzyme activity (Fig. 4).

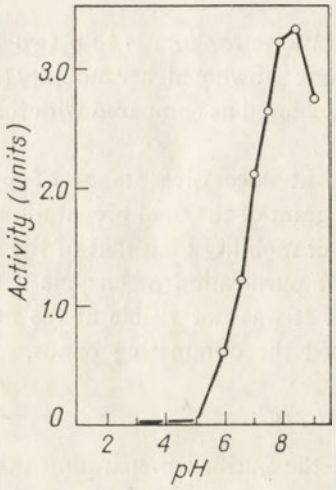


Fig. 3

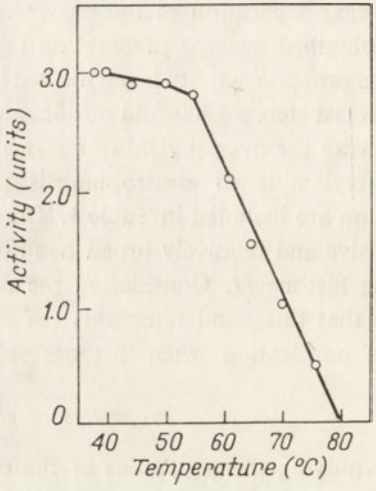


Fig. 4

Fig. 3. Plot of pH versus chromatin protease activity with total histone as substrate.

Fig. 4. Thermal sensitivity of chromatin protease. The activity of enzyme was determined at pH 8.0 after 5 min preincubation of the enzyme solution at indicated temperature.

The activity toward different proteins is shown in Table 2. Total histone and basic ribosomal protein were most susceptible to protease action whereas bovine albumin and lysozyme remained practically undigested.

Table 2

Hydrolysis of different proteins by protease from calf thymus chromatin

Samples were incubated at 37°C, pH 8.0, for 6 h.

Substrate	Activity	
	units	%
Total histone of calf thymus	3.00	100
Basic ribosomal proteins	2.88	96
Casein	1.83	61
Haemoglobin	1.38	46
Ribonuclease	0.45	15
Albumin	0.33	11
Lysozyme	0.00	0

It was earlier shown (Bartley & Chalkley, 1970; Kurecki *et al.*, 1971) that during the storage of chromatin preparation only two out of five histone fractions were degraded. In the present work, the specificity of the purified protease in histone degradation was studied by polyacrylamide-gel electrophoresis, and compared with the enzyme action in native chromatin (Fig. 5). In chromatin, histone *f1* was selectively degraded and after 4 h of incubation it disappeared completely, whereas histones *f2a1* and *f2a2* remained unchanged even after 24 h of incubation. On the other hand, the purified enzyme degraded all free histones practically uniformly showing no specificity toward any particular histone.

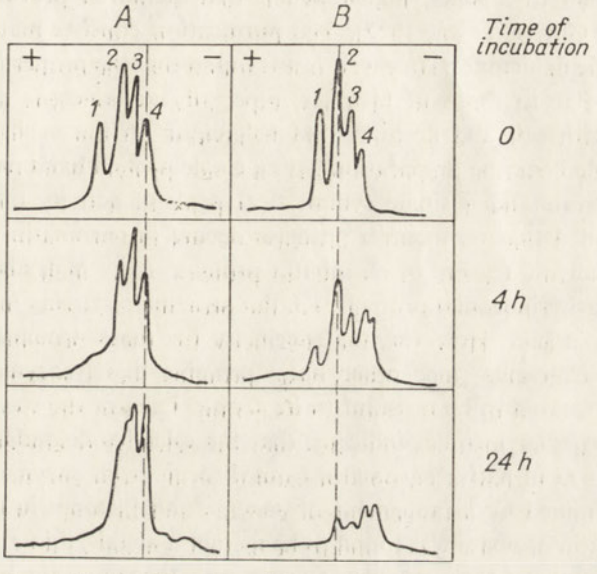


Fig. 5. Densitometer scans of polyacrylamide-gel electrophoresis patterns of histones and histone degradation products. *A*, Histones isolated after incubation at 37°C of freshly prepared intact calf thymus chromatin obtained as described by Marushige & Bonner (1966), except that sedimentation through 1.7 M-sucrose was omitted; the chromatin was suspended in 0.01 M-Tris-HCl buffer, pH 8.0, to a final concentration of about 2.5 mg of DNA/ml. *B*, Histones after incubation at pH 8.0 and 37°C of a system consisting of the purified enzyme and total histones at the same concentrations as in intact chromatin. *1*, histone *f1*; *2*, histones *f2b+f3*; *3*, histone *f2a2*; *4*, histone *f2a1*.

The purified chromatin protease had no effect on β-naphthylamides of alanine, glycine, arginine, lysine or histidine, which shows that the enzyme is not an exopeptidase. The protease showed no activity towards TAME or BTEE which proves that the purified preparation had no trypsin- or chymotrypsin-like esterase activity.

Preincubation of the isolated chromatin protease at 37°C and pH 8.0 for 10 min with 0.2 - 20 mM-EDTA or 0.2 - 5 mM-iodoacetamide had no effect on enzyme activity. The activity was not affected, either, by up to 5 mM-2-mercaptoethanol, thioglycollate, or 10 mM-sodium bisulphite. Insensitivity to sodium bisulphite indicates that the inhibitory effect of this compound on histone degradation in chromatin (Bartley & Chalkley, 1970) was not due to its direct action on enzyme activity.

The approximate molecular weight of chromatin protease was determined by gel filtration. The value of K_{av} for histone *f1* and lysozyme were 0.48 and 0.24, respectively, and that for chromatin protease, 0.38. Assuming a linear dependence between the K_{av} values and the logarithm of molecular weight, the molecular weight of chromatin protease was found to be $15\,400 \pm 1000$.

DISCUSSION

The described method allows to isolate from calf thymus chromatin a neutral protease, purified to a much higher extent than achieved previously (Kurecki & Toczko, 1972; Garrels *et al.*, 1972). The purification consists mainly in separation of the enzyme from histones. However, it was found that the properties of the protease are closely similar to those of histones, especially as concerns molecular weight, precipitation with salt and acetone, and behaviour on ion exchangers.

As the purified enzyme preparation gave a single protein band on polyacrylamide-gel electrophoresis and a single symmetrical peak of activity on gel filtration, it may be concluded that the neutral protease occurs in chromatin in a single form.

The characteristic feature of chromatin protease is its high ability to hydrolyse histones and basic ribosomal proteins, i.e. the proteins occurring in the cell in complex with nucleic acid. However, the specificity for these proteins is not due only to their basic character since other basic proteins like lysozyme and pancreatic ribonuclease appeared to be resistant to its action. Lack of the specificity of isolated enzyme for particular histones indicates that the selective degradation of only two histones *f1* and *f3* in native chromatin cannot result from enzyme specificity but is probably determined by arrangement of enzyme and histones in chromatin.

The chromatin protease was found to be neither a metal-, thio-, or acidic protease. In its pH optimum and high specificity for histones, the chromatin protease resembles neutral proteases from rat microsomes (Paik & Lee, 1970) and from rabbit polymorphonuclear leucocyte granule fraction (Davies *et al.*, 1971).

The occurrence of protease in chromatin from morphologically and functionally different animal tissues suggests that this enzyme is an important functional element of animal genome. It is involved more likely in essential processes of the cell than in physiological functions of particular tissues.

The characteristic high specificity of protease for histones and the known ability of histones to inhibit the template activity of DNA (Smith *et al.*, 1970; Hoare & Johns, 1971; Smart & Bonner, 1971), support the suggestion on the contribution of chromatin protease as an important element in the regulation of activity of animal genome.

The authors are grateful to Professor I. Chmielewska (Institute of Biochemistry, Warsaw University) for her encouragement and critical review of the manuscript, and to Professor K. Kleczkowski (Institute of Biochemistry and Biophysics, Polish Academy of Sciences) for making available the apparatus for preparative electrophoresis.

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OCZYSZCZANIE I CZĘŚCIOWA CHARAKTERYSTYKA PROTEAZY Z CHROMATYNY GRASICY CIELĘCEJ

Streszczenie

Opisano metodę oczyszczania proteazy z chromatyny grasicy cielęcej, umożliwiającą otrzymywanie preparatów enzymatycznych o około 1800-krotnie wyższej aktywności właściwej w porównaniu z wyciągiem z chromatyny oraz dających praktycznie pojedyncze pasmo białkowe w warunkach elektroforezy w żelu poliakrylamidowym. Oczyszczony enzym wykazuje, w stosunku do sumy histonów jako substratu, optimum pH około 8.5. Z największą szybkością hydrolizuje on histony i zasadowe białka rybosomalne, nie posiada aktywności esterazowej typu trypsyny i chymotrypsyny oraz nie wykazuje zdolności do hydrolizy β-naftyloamidowych pochodnych alaniny, glicyny, argininy, lizyny i histydyny. Aktywność enzymu nie jest hamowana przez związki chelatujące metale i blokujące grupy tiolowe, jak również przez kwaśny siarczyn sodu. Enzym posiada masę cząsteczkową $15\,400 \pm 1000$ daltonów.

Received 24 December, 1973.

ACTA BIOCHIMICA POLONICA

Vol. 21 1974 No. 2

RECENZJE KSIĄŻEK

T. Böszörményi, E. Csech, G. Gárdos and P. Kertai, TRANSPORT PROCESSES IN LIVING ORGANISMS. Akadémiai Kiadó, Budapest 1972; str. 349.

Przedstawienie w jednej, niezbyt obszernej książce wnikliwego przeglądu zagadnień transportu związków chemicznych i drobnych jonów przez błony biologiczne, jest zadaniem nadzwyczaj trudnym. Trudności te wynikają nie tylko ze złożoności i wielorakości poruszanych w książce problemów oraz różnorodności organizmów, u których badano procesy transportu przez błony (od bakterii i alg, poprzez rośliny wyższe aż do komórek i narządów zwierząt i człowieka). Są one także spowodowane olbrzymim bieżącym postępem, jaki się dokonuje w badaniu błon biologicznych, i wielką liczbą publikacji ukazujących się każdego tygodnia na tematy związane z odrębną już niemal dziedziną wiedzy nazywaną niekiedy "biomembranologią".

Autorzy omawianej monografii zebrali w jednym tomie podstawowe wiadomości, wraz z nader obszerną bibliografią, o błonach biologicznych, ich przepuszczalności i funkcjach transportowych w komórkach i tkankach roślin i zwierząt. Książka rozpoczyna się od ogólnej charakterystyki błon i procesów transportu, zdefiniowania zasadniczych pojęć przepuszczalności i sposobów penetracji przez błonę komórkową. Następna część nosi nazwę "część pierwsza"; zatytułowana jest "Procesy transportu w komórkach i tkankach roślin", a podzielono ją na dwa rozdziały. Pierwszy rozdział omawia procesy transportu u mikroorganizmów; uwzględniono tutaj transport nieorganicznych jonów i niektórych związków organicznych u bakterii, drożdży, pleśni Neurospora i alg. Drugi rozdział dotyczy procesów transportu w wyższych roślinach; omówiono w nim m.in. rolę ściany komórkowej, rolę mitochondriów i chloroplastów w wewnątrzkomórkowym transporcie jonów, znaczenie światła dla transportu jonów u roślin, a także transport przez korzenie roślin.

"Część druga", zatytułowana "Procesy transportu w komórkach i narządach zwierząt", jest wyborem tych zagadnień związanych z przepuszczalnością przez błony biologiczne, które są najbardziej reprezentatywne dla obecnego stanu wiedzy w tym zakresie. Omówiono więc tutaj procesy transportu przez błony erytrocytów, wydzielanie kwasu solnego przez śluzówkę żołądka, absorpcję z cienkiego jelita, funkcje transportowe nerki, skóry i pęcherza płazów, a także działanie gruczołu solnego u morskich ptaków. Ostatni rozdział części drugiej jest poświęcony procesom transportu w gruczole tarczowym, ze szczególnym uwzględnieniem transportu jodu.

Przyjęty przez autorów układ książki, z podziałem na procesy odbywające się u roślin i u zwierząt, nie był — jak każdy podział przyrodniczy — możliwy do konsekwentnego przeprowadzenia. I tak np. chociaż zagadnienie roli mitochondriów w wewnątrzkomórkowym transporcie omówiono w rozdziale poświęconym wyższym roślinom, to jednak większość cytowanych tam doświadczeń przeprowadzono na mitochondriach z tkanek zwierzęcych. Oczywiście nie ma to większego znaczenia dla czytelnika, który będzie chciał się zapoznać z podstawami zagadnienia, tym bardziej że książka jest zaopatrzona w obszerny alfabetyczny skorowidz rzeczowy i w skorowidz nazwisk.

Zarówno ze względu na bardzo szybki rozwój nauki o błonach biologicznych, jak i ze względu na niezbyt krótki cykl wydawniczy (autorzy piszą we wstępie, że upłynęły cztery lata od ukończenia manuskryptu do wydania książki), cytowane piśmiennictwo kończy się na roku 1968. Na końcu jednak — w postaci aneksu — dodano krótki wykaz najważniejszych pozycji piśmiennictwa do roku 1970. Tym niemniej omawiana monografia ma dużą wartość dla wszystkich tych, którzy

rozpoczynają pracę badawczą w którymś z licznych zagadnień "biomembranologii", a także dla tych badaczy, którzy eksperymentując nad jakimś wąskim problemem związanym z przepuszczalnością przez błony, chcieliby uzyskać szersze tło zagadnienia.

Książka wydana jest bardzo starannie, uchybienia korektorskie są bardzo nieliczne. Np. na str. 287 w równaniach dwukrotnie napisano "phosphatide" zamiast "phosphatidic", przeoczono również, że powinien być po lewej stronie równania (2) "lizophosphatidic acid" zamiast "phosphatidic acid", oraz "acylo CoA" zamiast "acetyl CoA". Zwyczaj używania skrótu: 1M w znaczeniu ilości jednego mola, a nie w znaczeniu stężenia jednomolowego (np. str. 286) jest także sprzeczny z ogólnie przyjętą konwencją.

Te drobne błędy nie przeszkodzą zapewne w dużym powodzeniu, jakim książka będzie niewątpliwie cieszyła się wśród biochemików, biofizyków i biologów innych specjalności.

Mariusz Żydowo

METHODICUM CHIMICUM. Band 1. "ANALYTIK" (F. Korte, ed.) George Thieme Verlag, Stuttgart — Academic Press, New York & London 1973; str. 1263, cena DM 496.

Oceniany tom o podtytule "Analityka" składa się z dwóch części i stanowi pierwszy z jedenastu tomów dzieła zatytułowanego "Methodicum Chimicum". Praca ta ma być krytycznym przedstawieniem metodologii chemicznej dla badaczy, jak i ludzi praktyki. Kierowana jest ona szczególnie do chemików wszelkiego rodzaju, jak i innych ludzi pracujących doświadczalnie w naukach przyrodniczych i medycynie, którzy zajmują się rozwiązywaniem międzydyscyplinarnych problemów badawczych i praktycznych metodami chemicznymi. "Methodicum Chimicum" ukaże się w trzech działach. Dział ogólny składa się z tomu pierwszego — analityka, drugiego — planowane syntezy i trzeciego — typy reakcji w procesach syntez. Dział systematyczny objęty tomami czwartym do ósmego dotyczyć będzie określonych syntez organicznych. W dziale specjalnym (tomy dziewiąty do jedenastego) zostaną przedstawione różne aspekty stosowania związków biologicznie czynnych.

Część pierwsza ocenianego tomu dotyczy metod wyosabniania oraz analitycznych metod stosowanych dla określenia struktury chemicznej. Opisano podstawowe zasady, standaryzację, optymalizację oraz sposoby liczbowego ujęcia wyników i sposób wykonania różnych metod rozdziału jak: destylacja, wirowanie, krystalizacja i rozdział wielofazowy, chromatografia bibułowa i cienkowarstwowa, chromatografia kolumnowa, absorpcyjna, jonowymienna, chromatografia gazowa i elektroforeza. W sposób przystępny przedstawiono metody wykazywania wiązań i funkcyjnych grup chemicznych przy użyciu analizy elementarnej, reakcji oksydo-redukcji, przyłączenia, podstawienia, przegrupowania, kondensacji, hydrolizy lub zobojętniania. Uwypuklono również znaczenie przekształceń chemicznych dla celów analitycznych. Syntetycznie — ale w sposób pozwalający ocenić ich przydatność dla badań związków chemicznych — są omówione metody spektroskopii w nadfiolecie i podczerwieni, magnetycznego rezonansu jądrowego, spektrografii masowej, polarymetrii, dichroizmu kołowego i dyspersji skręcalności optycznej. Natomiast nowe metody wysoce specjalistyczne, jak spektroskopia Ramana, neutronowa, paramagnetycznego rezonansu elektronowego, cyklotronowego rezonansu jonowego, jak i metody oparte na ugięciu promieni rentgenowskich, neutronów i elektronów z ich zastosowaniem dla badania struktury i ostatecznej konfiguracji, chociaż uwzględnione w książce szerzej, łącznie z aspektami teoretycznymi i praktycznymi, są dla czytelnika mniej przygotowanego trudne do oceny.

Druga część pierwszego tomu poświęcona jest metodom analitycznym o istotnym znaczeniu dla eksperymentalnych nauk biologicznych. Dokonano tutaj przeglądu szczególnych metod analitycznych służących do oznaczenia pierwiastków śladowych w materiale biologicznym. Cenne jest pokazanie najrozmaitszych technik specjalnych do rozwiązywania określonych problemów analitycznych, jak oznaczania pestycydów, środków leczniczych i spożywczych. W tej części tomu omówiono również w sposób syntetyczny podstawy teoretyczne metod, stosowaną aparaturę oraz techniki badawcze i ich ograniczenia metodyczne dla rozdziału i oznaczania węglowodanów, białek, peptydów i aminokwasów oraz kwasów nukleinowych. Czytelnik ma także możliwość

zapoznania się z metodami biochemiczno-biologicznymi uwzględniającymi zasady, na których opiera się analiza enzymatyczna, mikrobiologiczna oraz farmakologiczno-toksykologiczna. Tom pierwszy kończy się przedstawieniem warunków perspektywicznego rozwoju metod analitycznych w oparciu o udoskonalenia instrumentalne, sprzężenie kilku metod, ich automatyzację i komputeryzację. Ocena tomu nie byłaby pełna, gdyby nie zwrócono uwagi na walory cytowania na każdej stronie pozycji piśmiennictwa i bibliografii podstawowych opracowań z danej dziedziny. Wartości tomu pierwszego poświęconego analityce można dopatrzeć się również w tym, że wypróbowane przepisy metod z uwzględnieniem dyskusji przedstawiono tak, że nie zachodzi potrzeba sięgania do źródłowych pozycji literaturowych. Nowe natomiast drogi, które rozwiązują jakiś problem metodyczny w sposób bardziej nowoczesny, zostały przedstawione z uwzględnieniem zarówno aspektów teoretycznych, jak i praktycznych oraz zaopatrzone w ogromnie bogatą literaturę źródłową sięgającą dziesięciu tysięcy pozycji.

Tom pierwszy Methodicum Chimicum stanowi cenną pozycję dla wszystkich pracujących metodami chemicznymi chemików, przyrodników, lekarzy i farmaceutów.

Leon Żelewski

Cena zł 25.–

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Acta bioch. pol. Vol. 21, No. 2, s. 113—234, Warszawa, kwiecień—czerwiec 1974.
Indeks 35202