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CONTENTS

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Photo of Prof. W. Mozołowski by H. Nowak

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Professor Włodzimierz MOZOŁOWSKI

1895 — 1975

On May 6th, two days prior to his 80th birthday, Włodzimierz Mozołowski, M. D. passed away. He had been emeritus professor of biochemistry of the Academy of Medicine in Gdańsk, from which he also received the degree of *Doctor honoris causa*. During the period 1954 - 1968 he was Editor of *Acta Biochimica Polonica*.

W. Mozołowski was born in Sanok — a small town in southern Poland, at that time under the rule of the Austro-Hungarian Empire. There he obtained his general certificate of education with distinction in 1913. In the same year he registered as a medical student at the University of Lwów. At the outbreak of the first world war in 1914 the young student enlisted in the army organized by J. Piłsudski to fight for Poland's independence. He took an active and enthusiastic part in the war, at the end of which Captain Mozołowski, decorated with many military distinctions, returned to his studies and graduated in medicine at the Jan Kazimierz University in Lwów in 1922. He then joined the staff of the Department of Medical Chemistry in Lwów headed by J. K. Parnas, with whom he was associated until 1934.

The first research paper of W. Mozołowski, published in *Comptes rend. Soc. Biol.* in 1924, was concerned with the form of glucose present in the blood. He soon became passionately engaged in the research of the Parnas team on glycolysis and phosphate metabolism in the muscle. The main subject of research of W. Mozołowski at that time was ammonia formation in the muscle and in the blood. In a series of papers published mainly in *Biochemisches Zeitschrift* a detailed description is given of elaborate experiments leading to the discovery of 5'-adenylic acid as a source of ammonia formed in evasculated blood and in the working muscle. These experiments, and the conclusions presented in the papers, testify to the outstanding accuracy and criticism of the author.

Włodzimierz Mozołowski was a passionate teacher; his brilliant lectures were attended by several generations of veterinary, medical and other undergraduate and graduate students who appreciated very much the beautiful language and the ability to present chemical and biochemical problems in an exciting and understandable way.

After he had been endowed with *veniam legendi* at the University of Lwów in 1933, W. Mozołowski lectured for a short time at the Veterinary School in Lwów, and then was offered the Chair of Physiological Chemistry at the Stefan Batory University in Wilno, which he accepted in 1935. For four years, until 1939, when the University was taken over by the Lithuanian authorities, he lectured on physiological chemistry, and organized research, this time turning his interest towards detoxication

mechanisms in animal and in human subjects. This was, however, interrupted by the outbreak of the second world war. During the occupation professor Mozołowski remained in Wilno, working in a hospital laboratory and organizing secret Polish university education.

Shortly after the end of the second world war in 1945 he came to Gdańsk, which at that time was more a heap of ruins and burnt up buildings than a town in which academic life was to be revived. Here W. Mozołowski stayed for the rest of his life, which now was devoted entirely to the reconstruction of chemical and biochemical education and research. He was one of the very few Polish biochemists with academic qualifications who survived the war. He deserves credit for the initiation of normal teaching at the newly organized Faculty of Medicine of the Medical School in Gdańsk a few months after the end of the war. With great energy and enthusiasm, he not only lectured, organized practical classes, and tried to get laboratory equipment, but also endeavoured to reconstruct destroyed Polish scientific societies and scientific publications. He was very active in organizing the Polish Society of Physicians in Gdańsk, in encouraging contacts between all the biochemists in this country and between Polish and foreign biochemists. He was one of the founders of the Polish Biochemical Society and its first honorary member; and since the foundation of *Acta Biochimica Polonica* in 1954 until 1968 — Editor of this journal.

The greatest concern of professor Mozołowski was, however, the task of educating qualified research workers, which he felt was his most important responsibility in the rebuilding and development of science in Poland, destroyed by the six terrible years of the war.

One may feel it to be a kind of symbol that in this town, where the second world war started, the friendly personality of W. Mozołowski, his honesty and passion for biochemistry and for the truth, attracted many young people who gathered around him for twenty years until 1965 when he retired from the chair of biochemistry. Several research and teaching biochemical laboratories now functioning in Gdańsk are his achievement.

The community of Polish biochemists has lost its senior member, whose life was fulfilled with the noble fight for the highest values existing in human societies: for freedom, progress of science, truth and human dignity.

Mariusz Żydowo

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BEATA BRYSCH and M. CHORĄŻY

ISOLATION AND PROPERTIES OF POLY(A)-CONTAINING RNA FROM THE CYTOPLASM OF RAT LIVER CELLS*

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1. The described technique for preparative isolation of poly(A)-containing RNA by complexing with poly(U)-cellulose and elution at 45°C, is simple, reproducible, and the poly(U)-cellulose preparation is suitable for repeated use.

2. The poly(A)-containing RNA isolated from the poly(U)-cellulose complex is still contaminated by ribosomal RNA which can be removed by recomplexing. The purified preparation is heterogeneous on polyacrylamide-gel electrophoresis and is located over the region from 5-6S to 28S. Poly(A) segments of cytoplasmic RNA show heterogeneous size distribution.

3. In acidic medium, the poly(A) segments form ordered double-stranded structure.

4. The rapid labelling of rat liver and fibroblast cytoplasmic poly(A)-containing RNAs and their base composition resemble closely the corresponding properties of mRNA.

The discovery of poly(A) sequences in mRNA of eukariotic cells (Lim & Canellakis, 1970; Kates, 1970; Kates & Beeson, 1970) and in hnRNA (Darnell *et al.*, 1971a; Edmonds *et al.*, 1971; Lee *et al.*, 1971) permitted to establish the relationship between these two species of RNA (Jelinek *et al.*, 1973) and opened wide possibilities for studying the function of genome. The presence of a long poly(A) segment at the 3′ end of the mRNA molecule makes possible the obtaining of a highly purified mRNA preparation. The methods for isolation of poly(A)-containing RNA are most frequently based on hybridization with carrier-bound poly(U) or poly(dT) (Britten, 1963; Gilham, 1964; Edmonds & Caramela, 1969; Kates, 1970; Sheldon *et al.*, 1972; Singer & Penman, 1972). Another method consists of hybridization of RNA with poly(U) in solution and separation of the hybridized form from unreacted single-stranded RNA not containing poly(A) by hydroxyapatite chromatography

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

(Greenberg & Perry, 1972), adsorption on cellulose-acetate filters (Lee *et al.*, 1971) or adsorption on cellulose (Kitos *et al.*, 1972; Schutz *et al.*, 1972).

The present communication summarizes some observations on the methods of isolation and characteristics of the poly(A)-containing RNA from cytoplasm of rat liver cells.

MATERIALS AND METHODS

Reagents. [5-^{3}H]Uridine, code no. TRK 178 (spec. act. 24 Ci/mmole), [6-^{14}C] orotic acid, code no. TRK 171 (spec. act. 60.8 mCi/mmole) and [G-^{3}H]adenosine, code no. TRK 3 (spec. act. 9.6 Ci/mmole) were from The Radiochemical Centre (Amersham, Bucks., England). [^{32}P]Orthophosphoric acid, carrier-free, code no. RP-10 (spec. act. 37.8 mCi/mmole) was supplied by the Institute for Nuclear Research (Świerk, Poland).

Sodium dodecyl sulphate (SDS) and agarose were products of B. D. H. Chemicals Ltd (Poole, Dorset, England); Tris was from Sigma Chem. Co. (St. Louis, Mo., U. S. A.); tri-isopropyl naphthalene sulphonic acid, sodium salt (TNS) and polyvinylsulphate, potassium salt (PVS) were from Serva Feinbiochemica (Heidelberg, G. F. R.).

Poly(A) and poly(U) were products of Miles Chemical Co. (Elkhart, Ind., U.S.A.); cellulose, microgranular CC 41, and Whatman no. 1 filter paper were from Whatman Biochemical Ltd. (Maidstone, Kent, England); millipore filters HA were from Millipore Corp. (Bedford, Mass., U.S.A.).

Pancreatic deoxyribonuclease, electrophoretically pure, was from Worthington Biochemicals Corp. (Freehold, N. J., U.S.A.), ribonuclease A from Sigma Chem. Co. (St. Louis, Mo., U. S. A.), and ribonuclease T_1 from Sankyo Co. Ltd (Tokyo, Japan).

Acrylamide, *N,N'*-bisacrylamide, PPO, POPOP and ethyleneglycol were supplied by Koch-Light Lab. Ltd (Colnbrook, Bucks, England); 1,4-tetramethylenediamine (TEMED) by Fluka AG (Buchs, Switzerland), sucrose, RNase-free, by Schwartz-Mann (Orangeburg, N. J., U. S. A.), and actinomycin D by Calbiochem (San Diego, Calif., U. S. A.). Toluidine blue was from E. Merck (Darmstadt, G. F. R.), Sephadex G-100 from Pharmacia (Uppsala, Sweden), Eagle's medium from Difco Lab. (Detroit, Mich., U. S. A.). Calf blood serum was supplied by the Laboratory of Sera and Vaccines (Lublin, Poland). Other reagents were analytical grade products supplied by POCh (Gliwice, Poland).

Materials. The experiments were performed on rat liver, and on fibroblasts of rat embryos cultured in Roux flasks on the Eagle's nutrient medium fortified with 10% calf serum. Rats were of the Buffalo strain, weighing about 100 g; they were fasted for 18 h, killed under light ether anaesthesia and livers removed into cold solutions.

Labelling of cytoplasmic RNA. Fibroblasts were labelled with [^{3}H]uridine (5 - 10 μCi/ml) for 3 h; to arrest the synthesis of rRNA precursors, actinomycin D (0.05 μg/ml) was added to the medium 30 min prior to the addition of the label (Perry, 1963; Penman *et al.*, 1968).

Rats fasted for 18 h were injected intraperitoneally with actinomycin D (0.30 μg/g body wt.) to inhibit rRNA synthesis (Georgiev *et al.*, 1972). After 30 min the animals

were given intraperitoneally neutralized [^{32}P]orthophosphoric acid (40 - 70 μCi/g body wt.), or [^{3}H]uridine (25 μCi/g), or [^{14}C]orotic acid (6 - 20 μCi/g). Doubly-labelled RNA was obtained by injecting, in addition to orotic acid, [^{3}H]adenosine (30 - 50 μCi/g body wt.). The labelling proceeded for 3 hours.

Extraction of cytoplasmic RNA. The fibroblasts and liver were homogenized at 0-4°C in a glass homogenizer with a teflon pestle, by hand (fibroblasts) or mechanically (liver).

For extraction of cytoplasmic RNA, three procedures were used:

(i) The homogenate in 0.14 M-NaCl was extracted with phenol at pH 6 and 0°C (Georgiev *et al.*, 1962, 1972). To the interphase and phenol phase 0.14 M-NaCl was added and the mixtures re-extracted. The aqueous phases were combined, SDS was added to 0.5% (w/v), and the solution was deproteinized twice with a mixture of phenol (pH 6) and chloroform (1:1, v/v), and then twice with chloroform alone.

(ii) From the homogenate in 5 mM-KCl - 1 mM-$MgCl_2$ - 0.1 M-Tris buffer, pH 9, nuclei were centrifuged off for 15 min at 1600 *g* and 4°C (Penman, 1966). To the supernatant, SDS was added to 0.5% and the mixture extracted with phenol at pH 9 and 0 - 4°C (Lee *et al.*, 1971). The aqueous phase was deproteinized with a mixture of phenol (pH 9) and chloroform (1:1, v/v), and twice with chloroform. In some experiments, RNA was extracted from isolated polysomes, obtained according to Olsnes (1970).

(iii) From the homogenate prepared in 0.1 mM-EDTA - 0.1 M-NaCl - 1 mM-Tris-HCl buffer, pH 7.4 (Perry *et al.*, 1972), the nuclei were centrifuged off, to the supernatant TNS was added to 2% (Parish & Kirby, 1966), and RNA extracted with a mixture of phenol, pH 6 (saturated with 1 mM-EDTA - 0.1 M-NaCl - 1 mM-sodium acetate, pH 6) and chloroform (1:1, v/v). The aqueous phase was deproteinized twice with the same mixture (Perry *et al.*, 1972), then twice with chloroform. In some experiments, the interphase and phenol phase were re-extracted. In other experiments, RNA was isolated from the mitochondrial fraction and from the 12 000 *g* supernatant (Hirsch & Penman, 1973). To inhibit RNase activity, PVS (about 2 mg/litre) was added.

Preparation of poly(U)-cellulose and isolation of poly(A)-containing RNA. Poly(U)-cellulose was prepared according to Kates (1970) and Sheldon *et al.* (1972). Portions of about 1 g of a mixture containing 8 mg of poly(U) per 1 g of cellulose (microgranular CC 41) were suspended in 10 ml of 96% ethanol and irradiated with an Original Hanau Q400 germicidal lamp from a distance of 20 cm for 15 min.

Complexing on, and elution from poly(U)-cellulose of poly(A)-containing RNA or standard poly(A) were performed as described by Sheldon *et al.* (1972). The complexing was carried out in a plastic centrifuge tube. RNA or poly(A) was dissolved in the binding buffer (0.1 M-NaCl - 0.01 M-Tris-HCl, pH 7.5) and mixed with poly(U)-cellulose washed previously with the same buffer. After 1 h incubation at 0 - 4°C, the cellulose was sedimented by centrifugation (2500 rev./min, 5 min, 0 - 4°C) and washed several times with 10 ml (or larger) portions of the binding buffer. The RNA present in the supernatant and obtained by elution with the binding buffer is further referred to as the 4°C fraction or the non-bound fraction.

The cellulose-bound material was eluted with 5 - 10 ml portions of the eluting buffer (0.01 M-Tris-HCl, pH 7.5) stepwise at temperatures of 25° and then 45 - 50°C,

or only at 45 - 50°C. The fraction obtained by one-step elution is referred to as the 45°C or bound fraction.

Isolation of the poly(A) segment from cytoplasmic RNA. The cytoplasmic RNA from rat liver cells, doubly labelled with [^{3}H]adenosine and [^{14}C]orotic acid, was dissolved in 2 mM-$MgCl_2$ - 10 mM-Tris-HCl buffer, pH 7.5, and digested with pancreatic DNase (5 μg/ml) for 15 min at 37°C. Then KCl was added to a concn. of 0.3 M and the preparation treated for 15 min at 37°C with pancreatic RNase A (10 μg/ml) heated previously for 10 min at 90°C, and with RNase T_1 (50 units/ml). The digest was deproteinized with a mixture of phenol (pH 9) and chloroform as in procedure (ii) or with phenol (pH 6) - chloroform as in procedure (iii). The aqueous phase was mixed with 2.5 vol. of cold 96% ethanol, kept overnight at 2°C, and the precipitate formed was complexed with poly(U)-cellulose to separate the poly(A) segment.

Polyacrylamide-gel electrophoresis. Acrylamide and *N,N'*-bisacrylamide were recrystallized, respectively, from chloroform and acetone (Loening, 1967). The 2.1% polyacrylamide gel containing 0.5% agarose was prepared by the method of Peacock & Dingman (1967) as modified by Bourque & Naylor (1971). The slabs were about 8 cm long, current 3 mA, time 2 - 2.5 h, temp. 4°C, and the electrophoretic buffer was 0.05 M-Tris-phosphate, pH 7.6. The gels were rinsed with 1 M-acetic acid, then stained with 0.2% toluidine blue and destained with 1% acetic acid. For radioactivity determination, the slabs were sectioned into 1 mm or 2 mm slices. In the case of ^{32}P--labelled RNA, the radioactivity was counted as Čerenkov radiation from gel slices fixed on filter paper discs. When the RNA was labelled with ^{3}H or ^{14}C, the gel slices were dissolved in concentrated formic acid (0.2 ml/slice) by incubation overnight at 65°C, and the radioactivity counted in the scintillation fluid composed of PPO, POPOP, glycol and toluene, in the Packard 3380 scintillation counter.

Sucrose-gradient centrifugation. The 5 - 20% sucrose gradient was prepared either in 10 mM-Tris-HCl buffer, pH 7.5, or 50 mM-acetate buffer, pH 5.1, each containing 10 mM-NaCl, 1 mM-EDTA and 0.25% SDS. To the bottom of the tube was introduced 0.2 ml of 40% sucrose. The centrifugation was performed at 35 000 rev./min for 4.5 h at 20°C in a Spinco L-50 ultracentrifuge, SW 39 rotor. Fractions of 0.2 ml were collected from the bottom of the tube by syphoning and the radioactivity was determined as described above.

Melting profiles of the RNA fractions. Thermal denaturation of RNA was performed in the two buffers used for sucrose-gradient centrifugation, in a Beckman DU spectrophotometer, at a temperature increment of 2°C/min. Temperatures in the cuvettes were determined by means of a thermistor to an accuracy of 0.1°C. The RNA concentration ranged from 10 to 27 μg/ml.

RESULTS AND DISCUSSION

Properties of poly(U)-cellulose

Following u. v. irradiation of the mixture of 8 mg poly(U) with 1 g of cellulose, about 80% (6 -7 mg) of the starting amount of poly(U) became durably associated with cellulose (Table 1). Sheldon *et al.* (1972) using the same initial ratio of poly(U)

Table 1

Binding of poly(U) to cellulose and complexing of poly(A) with poly(U)-cellulose

The mixture of 8 mg of poly(U) and 1 g of cellulose in 10 ml of 96% ethanol was u.v. irradiated and centrifuged. The non-bound poly(U) was determined in the supernatant and in the indicated washing solutions. Then 200 - 600 μg of poly(A) was mixed with poly(U)-cellulose, and the binding capacity of the poly(U)-cellulose preparation for poly(A) was estimated.

Expt.	Non-bound poly(U) (%)				Cellulose-bound poly(U)		Bound poly(A), 45°C fraction (μg/g)
	in supernatant, room temp.	eluted with			%	mg/g	
		water, room temp.	binding buffer, room. temp.	eluting buffer, 45°C			
I	0.6	22.0	1.5	0.2	76.0	6.0	250
							220
II	0.8	6.0	4.0	0.2	89.0	7.0	120
							200
							160
							200
							140

to cellulose (Munktell cellulose), after irradiation with a 30 W Sylvania germicidal lamp obtained only about 5% of poly(U) bound to cellulose (0.3 - 0.4 mg/g). Nevertheless, the capacity of the two poly(U)-cellulose preparations for complexing poly(A) were closely similar, being 120 - 250 μg of poly(A) per 1 g of our preparation, as compared with 168 μg/g for the preparation of Sheldon *et al.* (1972). The difference in the amount of bound poly(U) may result from the application in our experiments of microgranular cellulose, and higher cross-linking of poly(U) due to irradiation with a lamp of presumably greater power. The high cross-linking could make a large part of poly(U) inaccessible for complexing with poly(A). This is supported by the weight ratio of poly(U) to bound poly(A), which in our experiments was 30 - 50, i. e. much higher than the corresponding value (2) reported by Sheldon *et al.* (1972).

However, it should be noted that the obtained preparation of poly(U)-cellulose is very stable and suitable for repeated use provided it is washed with the binding buffer.

Complexing of poly(A)

The conditions for binding of synthetic poly(A) to poly(U)-cellulose were checked using an excess of poly(A) or an amount equivalent to the complexing capacity of poly(U)-cellulose (Fig. 1a,b). In the presence of an excess of poly(A) (300 μg/g), most of poly(A) was not bound and was eluted with the binding buffer at 4°C; two bound fractions were eluted with the eluting buffer at 25° and 45°C. When the amount of poly(A) did not exceed the complexing capacity of poly(U)-cellulose (110 μg/g), only an insignificant amount of poly(A) was present in the 25°C fraction; almost whole poly(A) was stably bound and was found in the 45°C fraction.

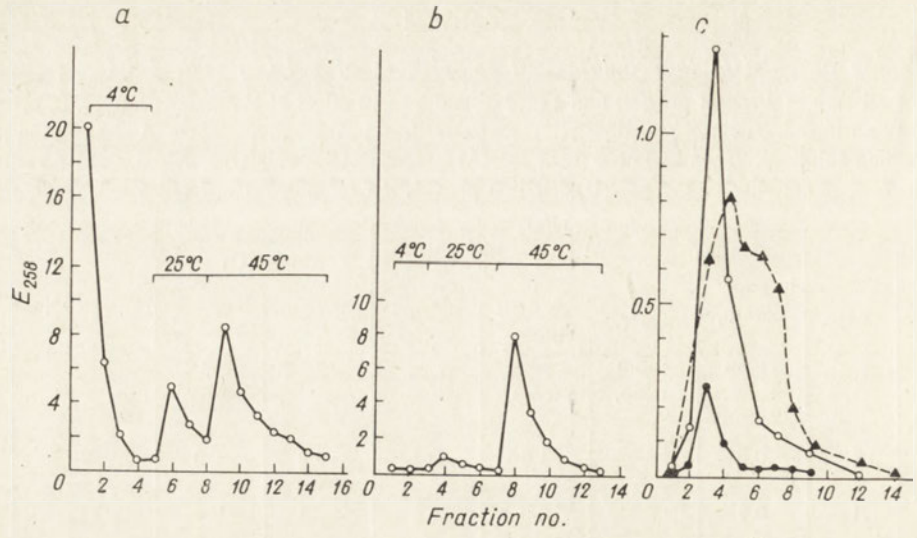

Fig. 1. Two-step elution of poly(A) complexed with poly(U)-cellulose: *a*, in the presence of excess poly(A) (300 μg/g of poly(U)-cellulose), and *b*, with an amount of poly(A) equivalent to the binding capacity of poly(U)-cellulose (110 μg/g). Elution at 4°C was performed with the binding buffer, and at 25° and 45°C, with the eluting buffer.
c, Sephadex G-100 gel filtration of the fractions obtained in Fig. 1a: ▲, non-bound fraction (4°C), ● and ○, bound fractions eluted, respectively, at 25°C and 45°C. The column was equilibrated with 20 mM-NaCl and eluted at room temperature with the same solution. Fractions of 3 ml were collected.

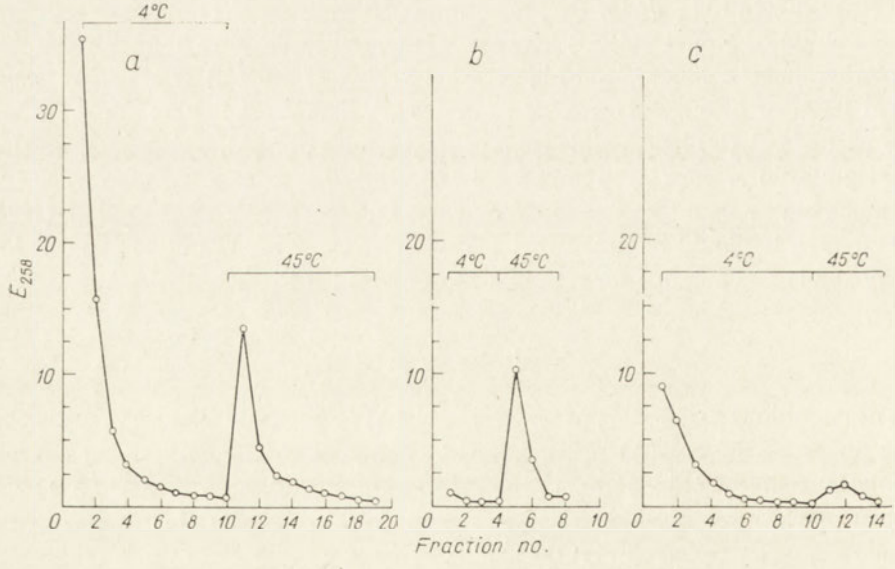

Fig. 2. One-step elution of poly(A) complexed with poly(U)-cellulose: *a*, with an excess of poly(A) (410 μg/g); *b*, at equilibrium to poly(U) (150 μg/g).
c, Elution of poly(A) complexed with cellulose alone (140 μg/g).

When the fractions obtained at the excess of poly(A) were submitted to Sephadex-gel filtration (Fig. 1c), fraction 4°C (non-bound) showed the presence of a greater amount of small molecules than the 25° and 45°C fractions which gave quite similar elution profiles.

In further experiments, after washing out the non-bound poly(A) with the binding buffer at 4°C, the bound poly(A) was eluted in one step at 45°C (Fig. 2a,b). When the complexing capacity of poly(U)-cellulose was not exceeded, the whole amount of added poly(A) became stably bound giving the 45°C fraction.

When cellulose alone instead of poly(U)-cellulose was used, under the same conditions only about 10% of poly(A) (16 μg/g of cellulose) was unspecifically bound to cellulose (Fig. 2c). Thus it appears that in our experiments poly(A) was predominantly bound to poly(U) cross-linked to the cellulose carrier.

Kitos *et al.* (1972) demonstrated that the cellulose non-bound to poly(U) or poly (dT) binds poly(A) or poly(A)-containing RNA at high salt concentration (0.5 M-KCl), or at low salt concentrations at a pH value of 5.5 or lower. It seems that, according to the reports of Rich *et al.* (1961) and Barszcz & Shugar (1964), under these conditions the double-stranded form of poly(A) is bound to cellulose.

Table 2

R_F *values of alkaline hydrolysates of poly(A) fractions eluted from poly(U)-cellulose*

The fractions of poly(A) eluted from poly(U)-cellulose were dialysed against water and subjected to alkaline hydrolysis (0.3 M-KOH, 37°C, 18h). The hydrolysate was neutralized with perchloric acid, applied to Whatman no. 1 paper and the chromatogram developed at room temperature for 40 - 45 h in the solvent system: butan-3-ol - water - concn. HCl (70:13.2:16.8, by vol.; Filipowicz, 1957). As standards, poly(A) and poly(U) subjected to hydrolysis under the same conditions were used. The spots were identified under a u.v. lamp.

Standard poly(U)	Standard poly(A)	Fraction 4°C	Fraction 25°C	Fraction 45°C
0.73	0.46	0.48	—	—
0.70	0.48	0.48	—	0.48
0.66	0.47	0.46	—	0.46
0.76	0.59	0.60	0.60	0.60

To check whether the elution of poly(A) is, or is not, accompanied by a release of poly(U), the eluted fractions were subjected to alkaline hydrolysis and analysed by paper chromatography (Table 2). The R_F values for the hydrolysates of all three fractions were practically identical and corresponded to the R_F for the hydrolysate of unfractionated poly(A); no spots with the mobility of poly(U) hydrolysate were observed.

Complexing of RNA

The elution of poly(A)-containing RNA from poly(U)-cellulose was performed in one step at 45°C (Fig. 3a). The complexing of this RNA with poly(U)-cellulose

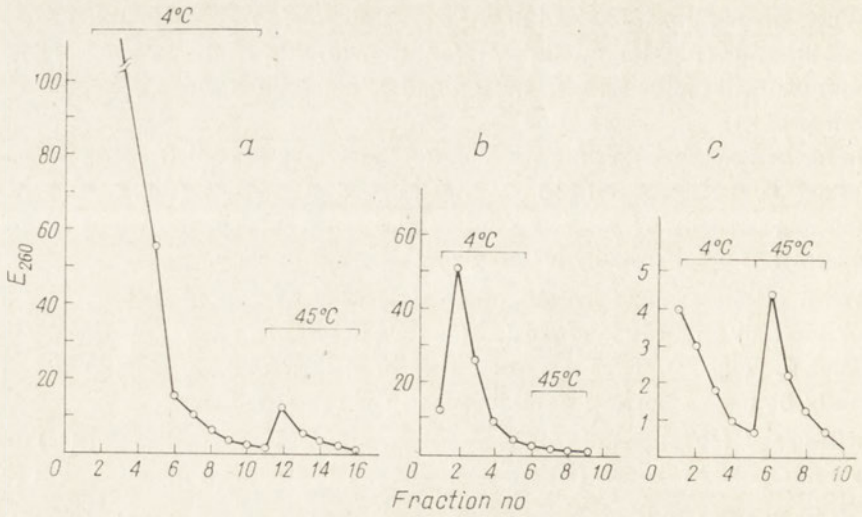

Fig. 3. Elution profiles of: *a*, total rat liver cytoplasmic RNA, obtained by procedure (iii), after complexing with poly(U)-cellulose; *b*, fraction 4°C after recomplexing; *c*, fraction 45°C after recomplexing.

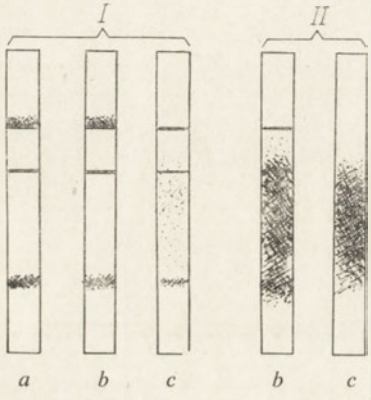

Fig. 4. Polyacrylamide-gel electrophoresis of cytoplasmic rat liver RNA after the first (*I*) and second (*II*) complexing with poly(U)-cellulose. *a*, Non-fractionated RNA; *b*, fraction 4°C; *c*, fraction 45°C.

Table 3

Complexing of poly(A)-containing RNA with poly(U)-cellulose, in comparison with its adsorption on millipore HA filters

Rat embryo fibroblast culture after incubation for 30 min with actinomycin D (0.05 μg/ml) was labelled for 3 h with [^{3}H]uridine (5 μCi/ml), then polysomal RNA was extracted as described in Methods, and nuclear RNA according to Georgiev & Mantieva (1962). RNA adsorption on millipore filters was performed according to Lee *et al.* (1971).

RNA	Radioactivity (%)	
	bound to poly(U)--cellulose	adsorbed on HA filters
Polysomal	21	15
Nuclear	42	36

Table 4

Purification of poly(A)-containing RNA by recomplexing with poly(U)-cellulose

The results are expressed as percentage of RNA E_{260} applied to the respective column. For details see Methods.

Source of RNA	Extraction procedure	Fraction 45°C	
		I complexing	Recovery on recomplexing
Rat liver	(i)	1.3	10
	(iii)	1.2	30
	(iii)	1.3	50
	(iii)	1.6	42

was compared with its adsorption on millipore-HA filters by the method of Lee *et al.* (1971); in both cases the amount of bound RNA was similar (Table 3).

The poly(A)-containing RNA eluted at 45°C from poly(U)-cellulose was contaminated with RNA fractions possessing the electrophoretic mobility of 28S, 18S and 4S RNA (compare Fig. 4, Ia,c, Fig. 8a,c) which could be removed by recomplexing. After recomplexing of the 45°C fraction, at the very most 50% was bound to poly(U)-cellulose (Fig. 3c, Table 4). The contaminating fraction removed by recomplexing (eluted at 4°C) consisted most probably of ribosomal RNA and degradation products (Fig. 4, IIb). On the other hand, the 4°C fraction after recomplexing was totally recovered at 4°C (Fig. 3b), and it contained mainly rRNA and 4S RNA.

Characteristics of the cytoplasmic poly(A)-containing RNA

For extraction of cytoplasmic RNA three procedures (see Methods) were tested to find that giving the least degradation and the highest yield of poly(A)-containing RNA. When unlabelled RNA was extracted from rat liver using procedure (iii), 1 - 2% of the material absorbing at 260 nm was bound to poly(U)-cellulose and was eluted at 45°C (Table 5, expts. 12 -14). This amount corresponds to the percentage of mRNA present in cytoplasm.

To obtain the highest labelling of the poly(A)-containing RNA (mRNA), a short time of labelling (3 h) and a low dose of actinomycin D were applied. On extraction of the labelled RNA, irrespective of the procedure applied, 10 - 30% of the radioactivity was bound to poly(U)-cellulose (Table 5). The preparations extracted by procedure (iii) showed the lowest contamination with proteins. Moreover, during the extraction by this procedure no losses were observed either in poly(A)-containing RNA or poly(A) sequences (Table 6).

The radioactivity patterns on polyacrylamide-gel electrophoresis of ^{32}P-labelled cytoplasmic RNA, isolated by the three procedures used, are presented in Figs. 5 - 7. The total RNA extracted by procedure (i) and (iii) showed three peaks of radioactivity corresponding to 4S, 18S and 28S RNA and a heterogeneous population

Table 5

Percentage of poly(A)-containing RNA in the cytoplasmic RNA

In isotopic experiments, actinomycin D was administered 30 min before the beginning of 3-h labelling, in doses of 0.05 μg/ml of fibroblast culture and 0.3 μg/g body wt. to rats. Cytoplasmic RNA was extracted by three procedures. For details see Methods. Except where indicated, total cytoplasmic RNA was studied. E_{260}/E_{280} ratio of the RNA preparations is also included.

Expt. no.	Source of RNA	Labelling	RNA bound to poly(U)-cellulose (%)		E_{260}/E_{280}
			E_{260}	c.p.m.	
	Extraction by procedure (i)				
1	Fibroblasts	[^{3}H]uridine[a]		5	2.0
2	Fibroblasts	[^{32}P]orthophosphate		32	1.6
3	Fibroblasts	[^{32}P]orthophosphate		11	1.9
4	Liver	None[c]	1.3[b]		2.4
	Extraction by procedure (ii)				
5	Fibroblasts (polysomes)	[^{3}H]uridine		21	1.9
6	Fibroblasts	[^{32}P]orthophosphate		10	1.3
7	Liver	[^{32}P]orthophosphate		17	1.7
8	Liver	[^{14}C]orotic acid		13	1.9
		+ [^{3}H]adenosine		14	
	Extraction by procedure (iii)				
9	Liver	[^{32}P]orthophosphate		28	2.0
10	Liver	[^{3}H]uridine	1.0[b]	10	2.0
11	Liver	[^{14}C]orotic acid	1.0[b]	11	2.3
		+ [^{3}H]adenosine		10	
12	Liver (12 000 *g* supernatant)	None[c]	0.9[b]; 1.4		2.0
13	Liver (12 000 *g* supernatant)	None[c]	1.2[b]; 1.0		1.9
14	Liver (mitochondria)	None[c]	1.5[b]; 1.8		2.0

[a] Labelling for 6 h. [b] Values obtained after precipitation of fractions 4°C and 45°C with ethanol. [c] In experiments without labelling, actinomycin D was omitted.

Table 6

Effectiveness of the first and second extraction by procedure (iii) of poly(A)-containing RNA or poly(A) segments from rat liver cytoplasm

	Total RNA extraction		Fraction 45°C extraction	
	1st	2nd	1st	2nd
[^{3}H]Uridine-labelled RNA, % of total ^{3}H radioactivity	95	5	10[a]	10[b]
Digestion product of RNA labelled with [^{3}H]adenosine and [^{14}C]orotic acid				
% of ^{3}H radioactivity bound to poly(U)-cellulose			98	2
% of ^{14}C radioactivity bound to poly(U)-cellulose			83	17
^{3}H/^{14}C ratio			26	8

[a] In relation to the amount after the 1st extraction.
[b] In relation to the amount after the 2nd extraction.

between 4S and 18S (Fig. 5a,c). The presence of radioactive 28S and 18S components indicates that despite the application of low doses of actinomycin D, partial synthesis of rRNA still occurred. The preparation obtained by procedure (ii) showed one peak corresponding to fraction 4S and heterogeneous material ranging from 4S to 18S, with a distinct preponderance of low-molecular species (Fig. 5b). No radioactivity corresponding to either of the two classes of rRNA was observed. Thus extraction at pH 9 (procedure ii) gives a partially degraded rRNA.

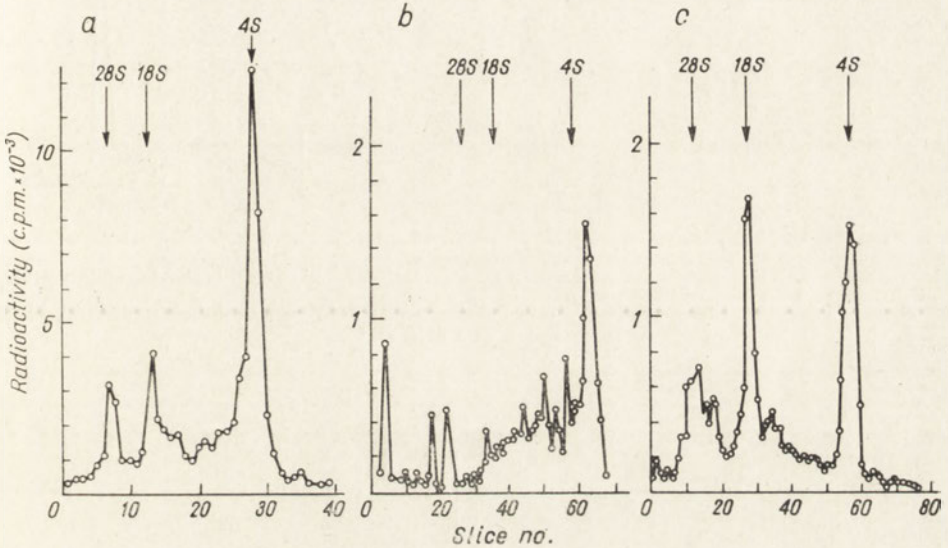

Fig. 5. Polyacrylamide-gel electrophoresis of the total rat liver cytoplasmic [^{32}P]RNA extracted by: *a*, procedure (i); *b*, procedure (ii); *c*, procedure (iii). Gel slices of 2 mm in *a*, and 1 mm in *b* and *c*. For details see Methods.

The non-bound to poly(U)-cellulose fraction 4°C of RNA isolated by procedure (i) and (iii) was composed mainly of 4S, 18S and 28S components (Fig. 6a,c). The preparation obtained by procedure (ii) showed only the component 4S and a heterogeneous region at higher S values (Fig. 6b).

On the electrophoretogram of fraction 45°C, the poly(A)-containing RNA was located in the region between 4S and 28S (Fig. 7). As mentioned above, fraction 45°C was often contaminated by ribosomal RNA, which could be removed by recomplexing with poly(U)-cellulose.

The radioactivity profiles of [^{3}H]adenosine-labelled cytoplasmic RNA extracted by procedure (iii) show that the 45°C RNA fraction was also heterogeneous (Fig. 8c) and distinct peaks corresponding to rRNA were present.

In neutral medium mRNA, in contrast to hnRNA, tRNA and rRNA, has no intramolecular regions with ordered secondary structure (Ryskov *et al.*, 1972; Jelinek & Darnell, 1972). On the other hand, in acidic medium the poly(A) homo-

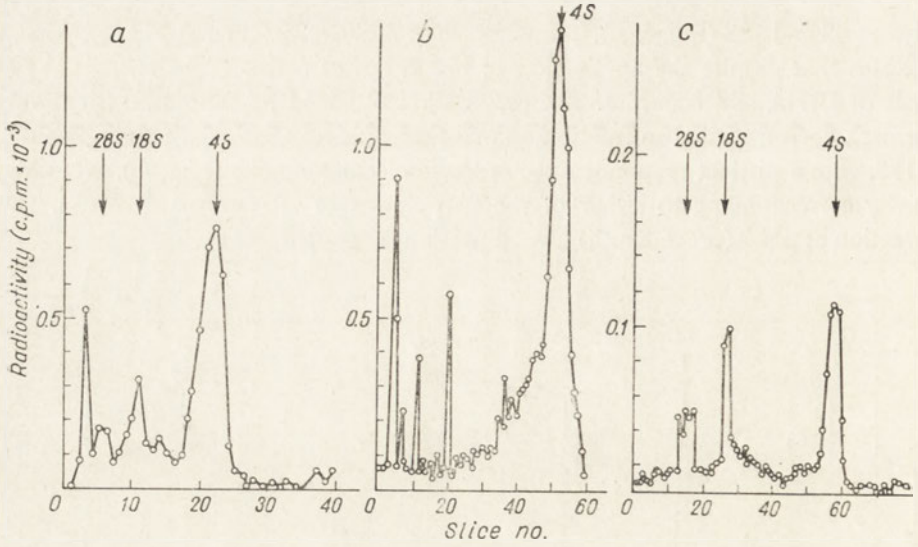

Fig. 6. Polyacrylamide-gel electrophoresis of poly(U)-cellulose fraction 4°C obtained from [^{32}P]RNA which was extracted by: *a*, procedure (i); *b*, procedure (ii); *c*, procedure (iii). Gel slices of 2 mm in *a*, and 1 mm in *b* and *c*.

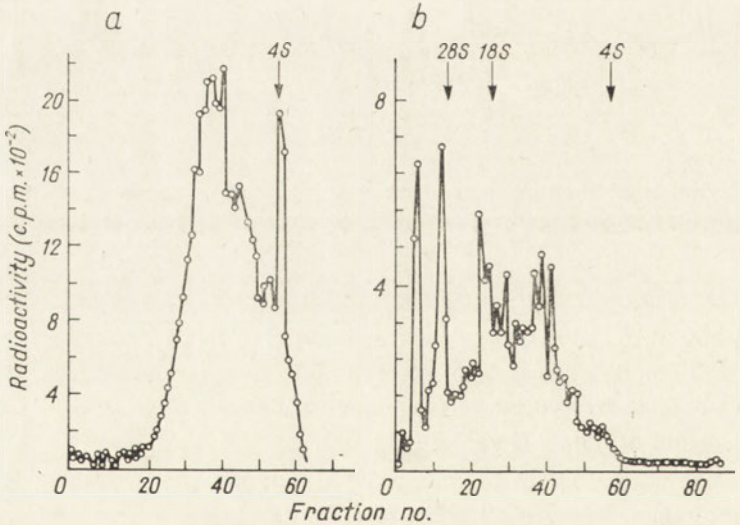

Fig. 7. Polyacrylamide-gel electrophoresis of poly(U)-cellulose fraction 45°C obtained from [^{32}P]RNA which was extracted by: *a*, procedure (ii); *b*, procedure (iii).

polymer is known to possess a double-stranded structure (Rich *et al.*, 1961; Barszcz & Shugar, 1964). This property of poly(A) segments could be utilized for rapid detection of poly(A)-containing RNA and its separation from other RNA species. Accordingly, both sucrose-gradient centrifugation of poly(A)-containing RNA was performed, and melting profiles obtained in weakly alkaline as well as in acidic media.

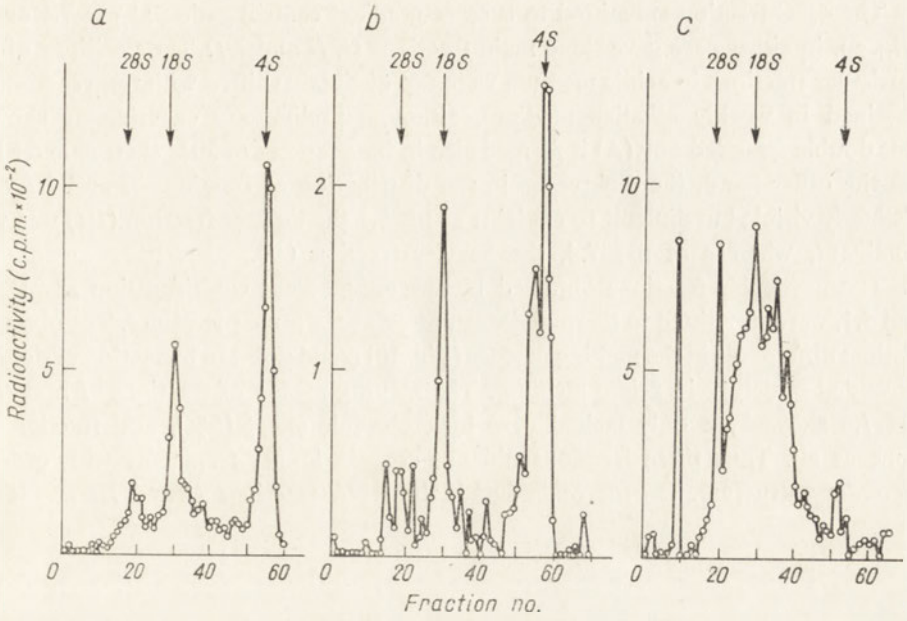

Fig. 8. Polyacrylamide-gel electrophoresis of [^{3}H]adenosine-labelled cytoplasmic RNA, extracted by procedure (iii). *a*, Total RNA; *b*, poly(U)-cellulose fraction 4°C; *c*, fraction 45°C.

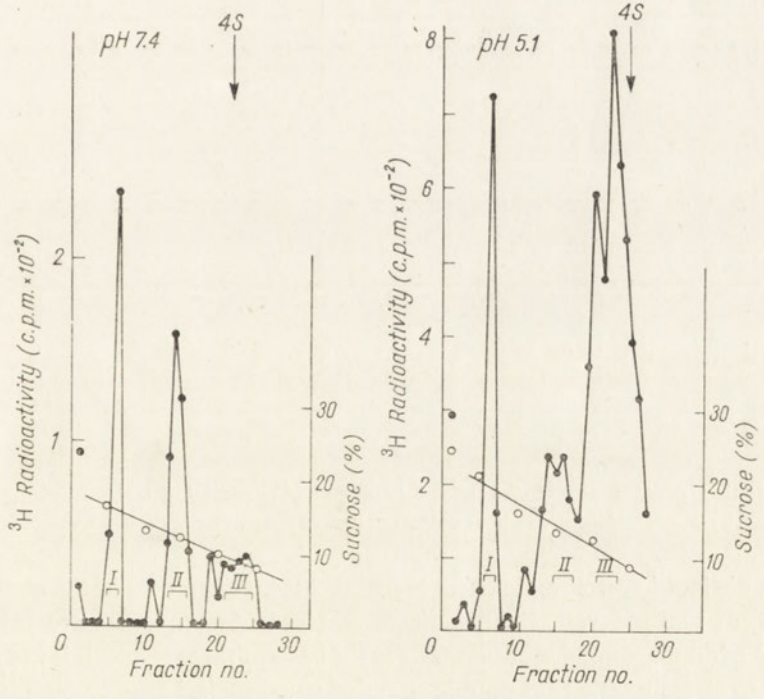

Fig. 9. Sucrose-density-gradient centrifugation at pH 7.4 and 5.1 of poly(A)-containing cytoplasmic rat liver RNA labelled with [^{3}H]uridine, extracted by procedure (iii). ●, Radioactivity; ○, sucrose gradient.

The 45°C fraction submitted to sucrose-gradient centrifugation at pH 7.4 or 5.1 (Fig. 9), in either case gave three main fractions (*I*, *II* and *III*). The positions of the particular fractions in acidic medium were not distinctly shifted with respect to those obtained in weakly alkaline medium, although preliminary experiments showed that double-stranded poly(A) is formed also in the presence of 20% sucrose and SDS. On the other hand, the differences in the distribution of fractions *II* and *III* were distinctly visible but difficult to explain: at pH 5.1 the lightest fraction (*III*) was predominant, whereas at pH 7.4, the heavier fraction (*II*).

The particular fractions obtained by sucrose-gradient centrifugation at pH 7.4 and 5.1, were submitted to thermal denaturation at the same pH values. The observed distinct differences in the melting profiles (Fig. 10) could point to formation of double-stranded structure in acidic medium. The melting profile of fractions *I* and *II* at pH 7.4 showed an only insignificant hyperchromic effect (5%), and fraction *III* none at all. Thus these fractions did not contain longer fragments with ordered secondary structure. On the other hand, the RNA fractions *II* and *III* at pH 5.1

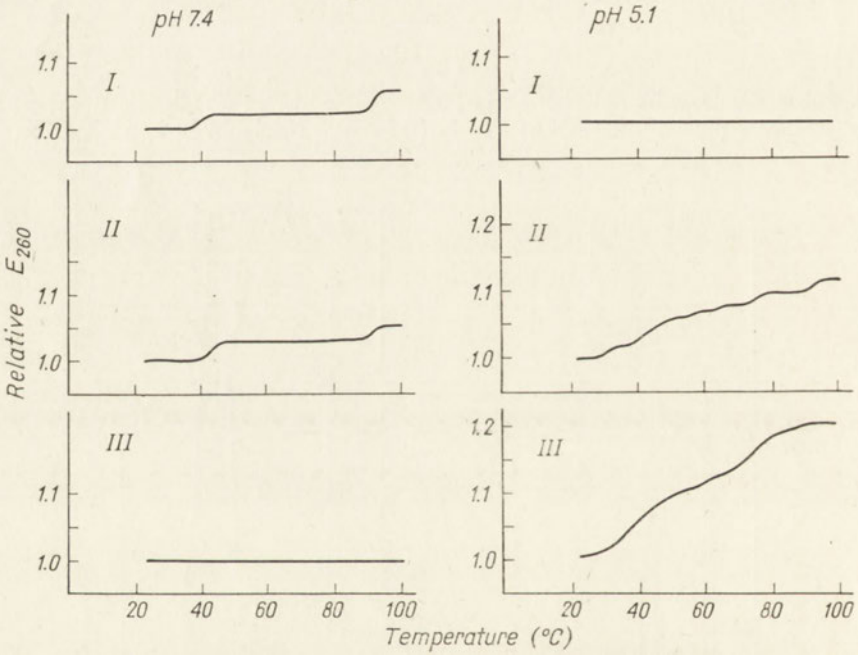

Fig. 10. Melting profiles at pH 7.4 and 5.1 of poly(A)-containing RNA fractions *I*, *II* and *III*, obtained by sucrose-density-gradient centrifugation at the corresponding pH values (see Fig. 9).

showed a distinct hyperchromic effect reaching 20%. Differences in the melting profiles at these two pH values can indicate that poly(A) sequences are involved in formation of double-stranded structures. On the basis of these results it is not possible, however, to assess whether the double-stranded structure is intra- or intermolecular. If the double-stranded structure were formed between poly(A) segments belonging to two different RNA molecules, one might have expected that the sedimentation

profile at pH 5.1 would be distinctly shifted, but no shifting was observed in our experiments.

The heaviest fraction *I* obtained at pH 5.1, in contrast to fractions *II* and *III*, showed no hyperchromic effect; this may be explained in two ways: either this fraction corresponds to the RNA containing no poly(A), or the poly(A) segment is so small that the hyperchromic effect is practically undetectable by the procedure used.

It seems that the induction of secondary structure of the poly(A) segments by low pH may be used as a marker of the 3′ end of the RNA chains. Additional studies of this effect with highly purified poly(A)-RNA are necessary.

Isolation and properties of poly(A) segment

The poly(A) segment was isolated from the doubly-labelled cytoplasmic RNA from liver cells extracted at pH 9 (procedure ii) or at pH 7.4 (procedure iii). The elution profiles from poly(U)-cellulose prior to and following RNA digestion with pancreatic and T_1 RNases, are presented in Fig. 11. As expected, poly(A), which is resistant to these two enzymes, was bound to poly(U)-cellulose and had the highest ratio of [^{3}H]adenosine to [^{14}C]orotic acid (Table 7). More poly(A) segments were obtained from the RNA isolated by procedure (iii) than (ii).

Table 7

Isolation of poly(A) segments from cytoplasmic RNA of rat liver cells doubly labelled with [^{3}H]*adenosine and* [^{14}C]*orotic acid*

For RNA extraction, procedures (ii) and (iii) were used. For details see Methods.

	Procedure				Procedure	
	(ii)		(iii)		(ii)	(iii)
	Radioactivity (%)				^{3}H/^{14}C ratio	
	^{3}H	^{14}C	^{3}H	^{14}C		
Total RNA	(100)	(100)	(100)	(100)	6	4
Fraction 4°C	86	87	90	89	14	4
Fraction 4°C of the digest	99	100	90	99	14	5
Fraction 45°C	14	13	.10	11	14	3
Fraction 45°C of the digest	1	0	10	1	82	88

The radioactivity profiles of electrophoretograms of the digestion products of doubly-labelled RNA (Fig. 12) indicate that poly(A) segments eluted as fraction 45°C were labelled practically exclusively with [^{3}H]adenosine and showed heterogeneous size distribution in the region above 4S (Fig. 12a). The material non-bound to poly(U)-cellulose appearing in the region of 4S as a sharp peak, was labelled both with [^{3}H]adenosine and [^{14}C]orotic acid (Fig. 12b). The observed heterogeneity of poly(A) segments resulted probably from their shortening during maturation of mRNA in the cell (Greenberg & Perry, 1972; Mendecki *et al.*, 1972; Sheiness & Darnell, 1973).

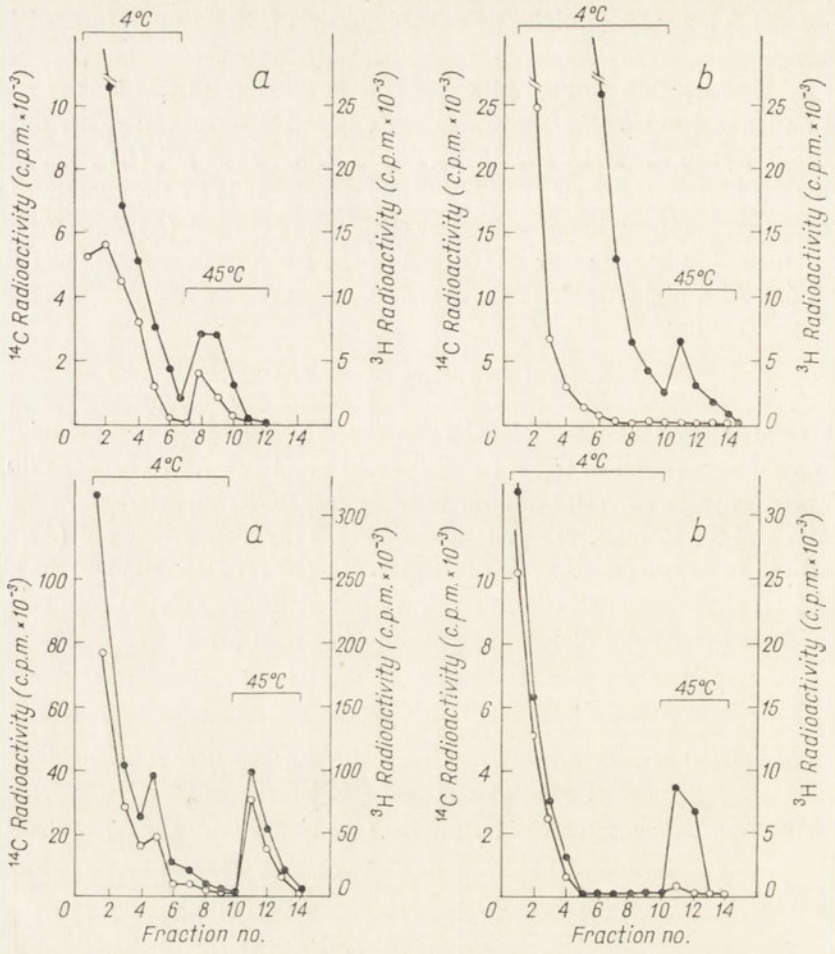

Fig. 11. Elution profiles from poly(U)-cellulose of cytoplasmic rat liver RNA labelled with [^{3}H] adenosine (●) and [^{14}C]orotic acid (○), *a*, before and *b*, after digestion by RNase A and T_1. Upper diagrams, RNA extracted by procedure (ii); lower diagrams, RNA extracted by procedure (iii).

The nucleotide composition of the cytoplasmic poly(A)-containing RNA from rat liver was found to be closely similar to that of mRNA of HeLa cells (Table 8), but different from the nucleotide composition of ribosomal RNA.

Concluding remarks

The presented technique for isolation of poly(A)-containing RNA is simple and adapted for preparative purposes.

From the three RNA extraction procedures tested, procedure (iii) permits to obtain the most effectively deproteinized and least degraded preparations with the highest content of poly(A) segments. The superiority of this procedure probably depends on the use of phenol-chloroform mixture at pH 7.4 according to Perry *et al.*

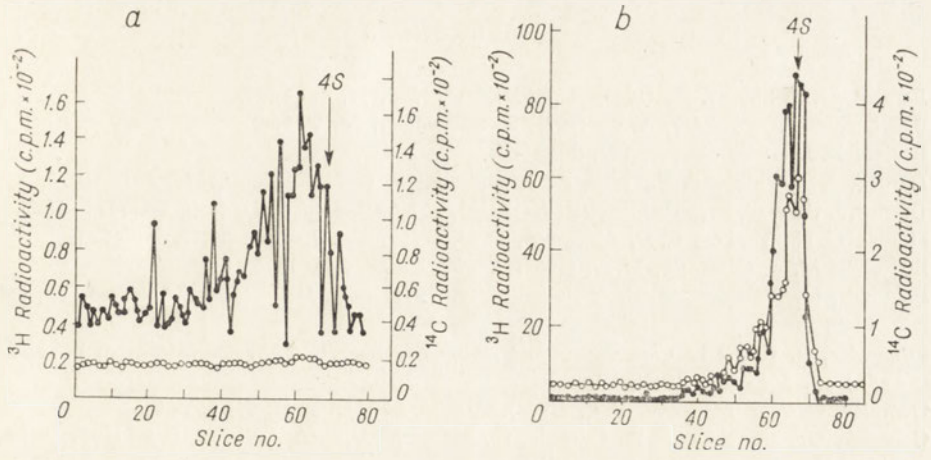

Fig. 12. Polyacrylamide-gel electrophoresis of RNA labelled with [^{3}H]adenosine (●) and [^{14}C]orotic acid (○), and digested by RNase A and RNase T_1. *a*, Fraction bound to poly(U)-cellulose; *b*, non-bound fraction.

Table 8

Nucleotide composition of cytoplasmic poly(A)-containing RNA from rat embryo fibroblast culture

The 45°C fraction from poly(U)-cellulose was obtained from ^{32}P-labelled cytoplasmic RNA by procedure (i). RNA was hydrolysed in 0.3 M-KOH, the ribonucleotides were separated by ion-exchange chromatography as described by Katz & Comb (1963), and the radioactivity was determined. The results are expressed as percentages.

RNA fraction	Nucleotide composition				GC	Reference
	AMP	UMP	GMP	CMP		
Cytoplasmic poly(A)-containing RNA from rat embryo fibroblasts	31.4	25.6	20.3	22.7	43.0	This paper
mRNA from HeLa cells	30.1	23.8	21.8	24.4	46.2	Darnell *et al.* (1971b)
rRNA from rat liver	19.5	20.5	34.5	25.5	60.0	Dessev *et al.* (1969)

(1972) in the presence of TNS, which is a more effective detergent and more potent inhibitor of RNase than SDS (Parish & Kirby, 1966).

The fraction of cytoplasmic RNA from embryonal fibroblasts and rat liver which complexes with poly(U)-cellulose, shares the properties of mRNA: it is rapidly labelled, contains poly(A) segments, is heterogeneous on polyacrylamide-gel electrophoresis, and has the characteristic nucleotide composition.

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IZOLOWANIE I WŁAŚCIWOŚCI RNA ZAWIERAJĄCEGO POLI(A) Z CYTOPLAZMY KOMÓREK WĄTROBY SZCZURA

Streszczenie

1. Technika preparatywnego izolowania RNA bogatego w poli(A) przy użyciu celulozy-poli(U) jest prosta, dająca powtarzalne wyniki, a preparat celulozy-poli(U) nadaje się do wielokrotnego stosowania.

2. RNA bogaty w poli(A) jednorazowo kompleksowany z celulozą-poli(U) zawiera jeszcze zanieczyszczenia rybosomalnym RNA, które można usunąć przez powtórne kompleksowanie. Oczyszczony preparat jest heterogenny i zajmuje po elektroforezie na żelu poliakrylamidowym rejon od 5-6S do 28S. Segment poli(A) w cytoplazmatycznym RNA jest heterogenny co do wielkości.

3. Sekwencje poli(A) w cytoplazmatycznym RNA tworzą w kwaśnym środowisku uporządkowane struktury dwupasmowe.

4. RNA bogaty w poli(A) izolowany z cytoplazmy komórek wątrobowych i fibroblastów szczura ma cechy (skład nukleotydowy i szybkie znakowanie w obecności niskich dawek aktynomycyny D) informacyjnego RNA.

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COMPLEXES OF DERIVATIVES OF 1-NITRO-9-AMINOACRIDINE WITH DNA**

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Substituted 1-nitro-9-aminoacridine derivatives were shown to inhibit RNA and to a lesser extent protein synthesis in cultured human cells. Complex formation between the compounds studied and DNA were considered to be responsible for their cytostatic action. Two types of complexes differing in their binding forces were found. The biological activity of the studied compounds seems not to be dependent on the existence of a positive charge on the acridine ring.

Some acridine derivatives exhibit biological activity and are used as potent antibacterial and antiplasmodial agents. Out of hundreds of the new acridine derivatives synthesized at the Department of Chemistry and Technology of Drugs, of the Polytechnic School in Gdańsk, 1-nitro-9-aminoacridine substituted in amino group by *N,N*-dimethylaminoalkyl exhibited strong cytostatic activity (Konopa *et al.*, 1969).

1-Nitro-9-[3′-(*N,N*-dimethyl)aminopropyl aminoacridine (the dye C-283) inhibited RNA biosynthesis in regenerating rat liver and cell cultures (Radzikowski *et al.*, 1967; Chorąży *et al.*, 1967), and it inhibited glycolytic and respiratory enzymes of the Ehrlich ascites cells (Gumińska, 1973). In the cells exposed to C-283 Vorbrodt (1970) observed "nucleolar segregation" and liberation of ribosomes from the endoplasmic reticulum.

Preliminary experiments pointed to the complex formation between DNA and C-283 under *in vitro* conditions. This reaction was reversible (as it could be judged on the basis of dialysis experiments) and it may be, at least partially, responsible for the biological activity of this compound. The influence of the positive charge on the

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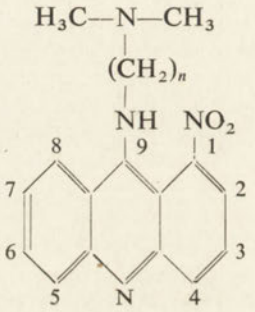
acridine ring on the ability of the dye to form complexes with DNA and on the biological effect of the acridine derivatives was the subject of the present work.

The problem has been already studied by other authors as it has theoretical and practical importance. The influence of the pK_a of the acridine derivatives on the binding constants of their complexes with DNA was studied by Löber (1967), and on the biological activity by Albert (1966) However, the compounds studied by those authors differed in the position and size of the substituents, so that the differences in their biological activity could be attributed to changes in properties other than pK_a values.

1-Nitro-9-aminoacridine derivatives at our disposal differed only in the size of the substituent at the 9 position of the ring (see Scheme 1). The different distance between the terminal amino group and amino group attached to the ring made these derivatives a good model for the study of the relationship between their biological activity and pK_a values. One could expect that the differences in the length of the side chain will exert an important influence on the activity of the derivatives in a biological system, although the observations of Drumond *et al.* (1965) indicate that this was not the case for the acridine derivatives having high and similar pK_a as 9-aminoacridine, atebrin etc., and the activity of which is thought to be based on the complex formation with DNA.

n	code number
2	C-337
3	C-283
5	C-609



Scheme 1

MATERIALS AND METHODS

Reagents. 1-Nitro-9-[2′-(*N*,*N*-dimethyl)aminoethyl]aminoacridine·2 HCl (C-337), 1-nitro-9-[3′-(*N*,*N*-dimethyl)aminopropyl]aminoacridine·2 HCl (C-283), and 1-nitro-9-[5′-(*N*,*N*-dimethyl)aminoamyl]aminoacridine·2 HCl (C-609), were synthesized at the Department of Chemistry and Technology of Drugs, Polytechnic School in Gdańsk. Highly polymerized calf thymus DNA was purchased from Worthington Biochemical Corp. (Freehold, N. J., U. S. A.).

DL-[^{14}C]Phenylalanine (spec. activity 2.6 mCi/mmole) and [^{3}H]uridine (spec. activity 13 mCi/mmole) were products of The Radiochemical Centre (Amersham, Bucks., England).

PPO and POPOP were products of Koch-Light (Colnbrook, Bucks., England). All other chemicals were of analytical grade, and were purchased at POCH (Gliwice, Poland).

Material. KB cells originating from human larynx cancer grown in the Eagle's medium, were kindly supplied by Dr. J. Konopa of the Polytechnic School in Gdańsk.

Incorporation of labelled precursors into RNA and proteins. Samples of the suspension of KB cells in Eagle's medium were distributed into Carrel's flasks and allowed to grow for 24 hours. The medium was then removed and to the cells was added fresh Eagle's medium containing 1 μCi/ml of labelled precursor (either DL-[^{14}C] phenylalanine or [^{3}H]uridine) and 1 μg/ml of the dye studied, and the mixture incubated for 30 min at 37°C. The collected cells, after being washed with prewarmed Hanks solution and 0.15 M-NaCl, were frozen on solid CO_2, thawed, and disrupted by sonication. In the case of protein labelling, 1 ml of 5% ice-cold trichloroacetic acid (TCA) was added per 5 ml of cell homogenate. The precipitate sedimented at 1500 *g* for 15 min was washed three times with 5 ml of cooled 5% TCA and dissolved in 0.2 ml of 95% formic acid (10 min at 80°C), mixed with 10 ml of scintillation solution (3 g PPO + 1 g POPOP + 300 ml 2-methoxyethanol + toluene to 1000 ml), and counted in Tracerlab Corumatic 200 scintillation counter. In the case of RNA labelling with [^{3}H]uridine, cold $HClO_4$ at final concentration of 0.5 M was used instead of TCA.

To the control cultures, acridine derivatives were not added.

Dialysis. To check the reversibility of dye-DNA complex formation, an exhaustive dialysis was performed. The dialysis was done at pH 5.0 for C-283, and at pH 7.5 for C-337. At acid pH the dye was in bivalent form and at pH 7.5 in monovalent cationic form, as the proton from ring nitrogen had been dissociated. DNA, 500 μg/ml, and the dye, 40 μg/ml, were dissolved in 0.01 M-phosphate buffer of appropriate pH, and 5-ml portions were placed in dialysing bag (Serva, Heidelberg, G. F. R.) and dialysed at 4°C against 1000 ml of the same phosphate buffer changed every 24 hours. At 24 h intervals samples of the solution were withdrawn and the absorption was estimated at the dye absorption maxima (450 nm for C-283, and 400 nm for C-337) using Unicam SP-500 (Series 2) spectrophotometer. Dialysis was conducted until disappearance of the absorption. The same series of experiments were performed in 0.1 M-phosphate buffer.

Binding curves. In DNA-dye binding experiments the DNA sample (50 μg/ml) dissolved in appropriate phosphate buffer, was titrated with concentrated solution of the dye, added in portions of 2 μg/ml. After each addition, the absorption at 450 nm (for C-283) or at 400 nm (for C-337) was measured.

The simultaneous measurements of changes in the visible spectrum of the dye in the course of titration permitted to construct binding curves. If the dye reacts with the DNA nucleotide in a reversible mode and if only one kind of complex is formed, one can write:

$$\mathrm{DNA} + r\mathrm{A} \overset{K}{\rightleftarrows} \mathrm{A}_r\mathrm{DNA}$$

where: A — molecule of acridine derivative, and r — number of the dye molecules bound to one nucleotide.

The binding constant K was determined from the Scatchard plot (Müller & Crothers, 1968). From the law of mass action the following equation could be formulated:

$$\frac{C_K}{C_{DNA} \cdot C_F} = f \frac{C_K}{C_{DNA}}$$

where: C_K — concentration of the dye bound to DNA (moles/l),
C_{DNA} — concentration of the DNA (moles of nucleotides/l),
C_F — concentration of the free dye (moles/l).

The concentration of the free dye was determined from the equation:

$$C_F = C_A - C_K$$

where: C_A — total concentration of the dye (moles/l).
The total amount of the dye (C_A) was known as we added the dye to DNA solution from the dye stock solution prepared just before the experiment. The aqueous stock solution of the dye was distributed in equal samples into vials; the samples were subsequently lyophilized and kept in a desiccator over P_2O_5, in the dark. For each series of experiments the dye was redissolved in appropriate buffer directly before use. The concentration of the dye in the complex dye-DNA (C_K) was experimentally established from the extinction of the solution and the molar extinction coefficients for the free (ε_F) and bound (ε_K) dye:

$$A = C_K \varepsilon_K + C_F \varepsilon_K$$

where: A — total extinction of the solution.
The molar extinction coefficient of the complex was determined for the solutions which contained an 10 to 100-fold excess of DNA so it could be assumed that all molecules of the dye were bound to DNA. The binding constant was determined from the slope of the curve and the number of the dye molecules bound per one nucleotide as an intersection point of extrapolated curve with the C_K/C_{DNA} axis. This method can be applied when only one kind of complex is formed, when the binding sites are equal stereochemically and energetically, and no cooperativity effects of binding take place. As the dyes studied could be present in a solution in two forms, we chose such pH at which only one form was present and the influence of the second form was negligible.

Determination of pK_a *values.* Acid-base equilibrium constants were established from the data of spectral titration of a free dye and dye bound to the DNA. The pH (Radiometer pH-meter, model PHM-22) and the absorption at 450 nm of the sample in phosphate buffer were measured and the pK_a determined from the plot of absorption *versus* pH. pK_a values of the complexes were determined for the solution containing a 20-fold excess of DNA, assuming that at this excess all dye molecules are bound. The titration curves were established also by potentiometry: 5 mg of the dye in 2 ml of bidistilled water was titrated with 1 M-HCl or 1 M-NaOH using a micropipette.

Melting profiles of DNA and dye-DNA complexes were determined at 260 nm in a Beckman DU spectrophotometer equipped with a thermostated cuvette holder.

The concentration of DNA was 24 μg/ml, and that of the dye 2 μg/ml of 0.01 M-phosphate buffer, pH 7.0. The amount of the dye bound to DNA was calculated from the extinction at 400 nm using the previously established extinction coefficients of the dye and the complex. Under the conditions used, spectra of the complexes in the visible range remained reasonably constant at the temperatures below the melting point.

The theoretical melting temperature was calculated from the formula given by Chambron *et al.* (1966) which was obtained on the assumption that the increment in the T_m of DNA after binding with the charged dye results from the screening effect of the dye on the phosphate backbone. The mutual repulsion of the phosphate groups diminishes, and the T_m grows higher.

$$\Delta T_m = \frac{e\lambda\Psi}{\Delta S_0} \cdot 2r'$$

where:

r' — is the number of the dye molecules charged in the ring bound to one nucleotide residue;

$e\lambda$ — is a part of the electrostatic charge which interacts with the neighbouring charges in double helix;

Ψ — is the electrostatic potential of NH^+ group in the heterocyclic ring of the dye;

ΔS_0 — is the standard entropy change;

ΔT_m — is the increment of melting temperature of the DNA after dye binding. The numerical values of Ψ, $e\lambda$ and ΔS_0 have been given by Chambron *et al.* (1966), and we have used them without changes; r' has been calculated from the applied dye to DNA ratio, and pK_a of the complexed dye. $r = C_K/C_{DNA}$ was determined in the same manner as in the binding experiments.

The dye is bound to DNA partially as a bivalent and partially as a monovalent cation. In the solution in which $pH = pK_a$ of the complex both forms are present in equal quantities, $r' = 0.5\,r$. For the complex in the solution in which pH differs from pK_a, r' can be obtained from the spectral titration curve.

Under the experimental conditions used (0.01 M-phosphate buffer, pH 7.0) the value calculated from the formula is:

$$T_m = 65.9\,r'\,(°C)$$

RESULTS AND DISCUSSION

All the three acridine derivatives studied, significantly suppressed RNA and protein synthesis in the cells grown in a culture (Table 1). During 30 min of incubation with the dye, the incorporation of [^{3}H]uridine into RNA was more suppressed than the incorporation of [^{14}C]phenylalanine into protein. On 30-min incubation, the synthesis of cell protein appeared to be less susceptible than that of RNA, and this could indicate that the inhibition of RNA synthesis was the primary event. No distinct differences in the inhibitory effect were observed between the three dyes

Table 1

Inhibition of the synthesis of RNA and protein in KB cells by 1-nitro-9-aminoacridine derivatives

KB cells were incubated for 30 min with [^{3}H]uridine or [^{14}C]phenylalanine in the presence of the indicated dye. The results are expressed as c.p.m./10^6 cells; each figure represents an average value from at least three cell cultures and triplicate radioactivity counting.

Derivative*	pK_a	[^{3}H]Uridine Experiment			Inhibition (%)	[^{14}C]Phenylalanine Experiment			Inhibition (%)
		I	II	III		I	II	III	
None, control		402	473	548		386	402	187	
C-337 (2)	4.75	19	15	21	96	203	137	91	55
C-283 (3)	6.2	22	14	17	96	141	189	134	52
C-609 (5)	7.4	27	31	29	93	178	262	169	37

* The number of carbon atoms in the side chain of each dye is indicated in parentheses.

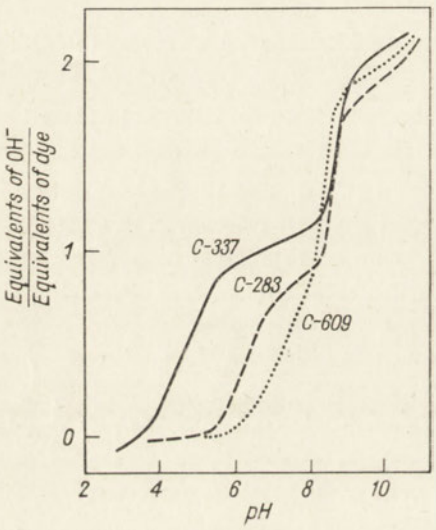


Fig. 1. Potentiometric titration of acridine derivatives by alkali. To 5 mg of the dye dissolved in 2 ml of water, 1.0 M-NaOH was added gradually and after each step pH was measured. The first and the second transition correspond to ring imino and chain amino group dissociation, respectively.

differing in the number of carbon atoms in the side chain. It was found previously (Radzikowski *et al.*, 1967) that C-283 showed a potent inhibitory effect on the synthesis of all classes of cellular RNA, as well as a distinct effect on cell ultrastructure (Vorbrodt, 1970).

In a solution with ionic strength and pH similar to physiological, 1-nitro-9-aminoacridine derivatives exist as mono- and bivalent cations. The changes of pH during titration of the three dyes with 1 M-HCl or 1 M-NaOH (Fig. 1) gave the plots with two inflection points in the pH region 3 - 10. The pK_a values corresponding to these inflections are given in Table 2. As the polymethylene side chain becomes longer, the distance between two charged nitrogen atoms increases. This results in the decrease of the repulsion forces between the protons and lessening of the ability

Table 2

pK_a values of the dyes measured by spectral and potentiometric titration

Spectral titration was performed in 0.01 M-phosphate buffer, and potentiometry in water solution.

Derivative	Spectral titration		Potentiometry	
	free dye pK_a	dye bound to DNA pK_a	free dye pK_{a1}	free dye pK_{a2}
C-337	4.75	6.0	4.75	8.8
C-283	6.2	7.4	6.45	8.8
C-609	7.4	—	7.4	8.55

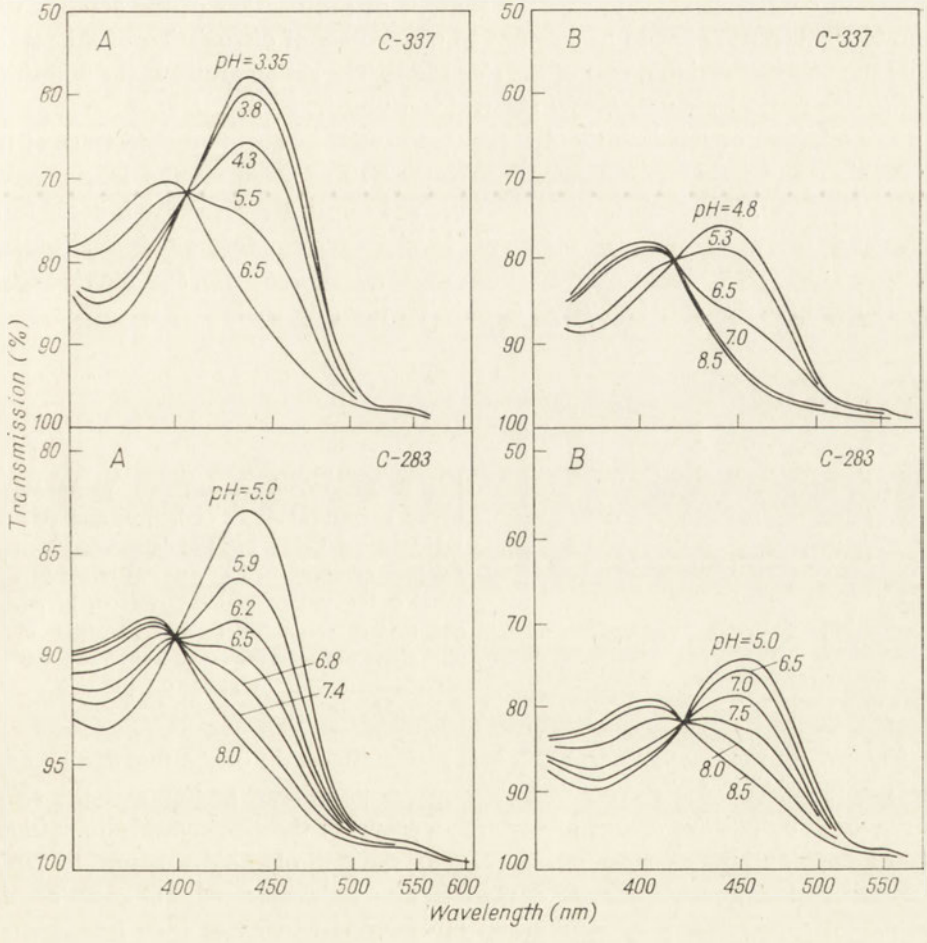


Fig. 2. Spectra of C-337 (upper diagrams) and C-283 (lower diagrams): *A*, free and *B*, bound to DNA, at different pH values. The final concentration of DNA was 200 μg/ml, of C-337 10 μg/ml, and of C-283 in *A* 5 μg/ml and in *B* 10 μg/ml; 0.01 M-phosphate buffer of the indicated pH was used.

of the *N*-imino proton to dissociate. Thus the dissociation constant for the proton on ring nitrogen increases. When the imino proton is dissociated the dye molecule retains only the amino proton for which the dissociation constant, about 8.5, is similar for all three derivatives studied.

The spectra for the compounds C-337 and C-283 in the solution differing in pH, are shown in Fig. 2A. At the pH at which both compounds have only one charge, the maximum for C-337 was at 396 nm and for C-283 at 393 nm. At low pH where the dyes are in the form of bivalent cations, the absorption maximum was at 440 nm for both compounds. The pH range of the transition from one form to another is different for the two compounds studied. Their pK_a values determined by spectral titration are similar to the pK_{a1} values determined potentiometrically (Table 2). As the absorption in the visible region for the acridine dyes can be ascribed to the acridine ring, the changes with pH of the position of the maximum and the extinction coefficient indicate that the changes of protonation of the acridine ring take place. The pK_{a2} has to be ascribed to the process of dissociation of the proton from the terminal amino group of the side chain. The amino group at the 9 position of the dye ring has a pK_a below the region studied.

The addition of DNA to the dye solution caused a pronounced decrease of the absorption and a shift of the maxima by about 10 nm towards the longer wavelength (Fig. 2B). These effects were observed at low pH where the dye is in the form of bivalent cation, as well as at higher pH in which the dye forms only monovalent cations. Thus DNA binds to the dye both when the acridine ring and the side chain are charged, and when the charge is only on the side chain.

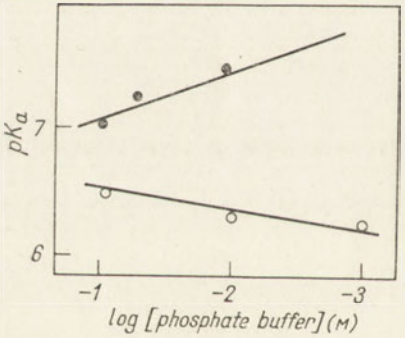


Fig. 3. Dependence of pK_a on phosphate buffer concentration of (○), free, and (●), DNA-bound C-283. pK_a was determined from the plot of absorption at 450 nm *versus* pH. Final concentration of C-283 was 40 μg/ml, and that of DNA 500 μg/ml. The pH of phosphate buffer in the range from 5 to 9 was raised stepwise by about 0.5 pH unit.

The pK_a of the dye bound to DNA is higher than that of the free dye because the bound molecule is surrounded by negative phosphate groups and the effect of repulsion by positive charge of the amino group of the side chain is diminished. With increasing buffer concentration (Fig. 3), the pK_a of the dye bound to DNA decreases and approaches the pK_a of the free dye. This effect could be explained by the shielding of DNA phosphate group by counterions so that their influence on the side-chain proton is diminished.

Both for monovalent and bivalent cation forms of the dye, the reversibility by dialysis of complex formation was demonstrated spectrophotometrically. This

makes possible the application of the Scatchard's method to a study of the binding process, provided that only one ionic form of the dye is present in the solution. The binding curves for C-283 at pH 4.55 (bivalent cation) and for C-337 at pH 7.0 (monovalent cation) were linear only over certain ranges of $r = C_K/C_{DNA}$ (Fig. 4). As the r grows the slope of the plot decreases. This indicates that the DNA-dye complex formation could not be described by a simple equation with one equilibrium constant, and points to the existence of two or more binding sites, and two or more types of dye-base or dye-dye interactions. The tangent of the linear part of the plot determined for the low amount of dye in the complex, diminished as the buffer concentration grew higher. This effect took place for both forms of the dye. The binding diminished with the increase in ionic strength, nevertheless even in 0.1 M-phosphate buffer the complex was not completely dissociated. The existence of the positive charge on the acridine ring had surprisingly little influence on the complex formation.

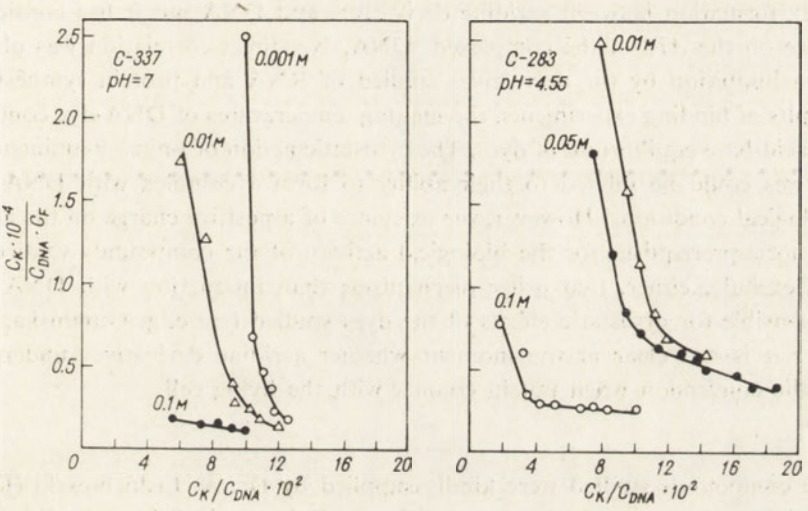


Fig. 4. Binding to DNA of the dye in monovalent cationic form (C-337, pH 7.0) and bivalent cationic form (C-283, pH 4.55). The dye from the stock solution was added portionwise to DNA in phosphate buffer of indicated concentration, and after each addition step the extinction at 400 nm was measured. For details see Methods.

The melting temperatures of the complexes of DNA with three acridine dyes are shown in Table 3. The experimentally established temperatures were compared with those calculated theoretically. According to Chambron *et al.* (1966), the increase in the stability of the double helix caused by complex formation is due to the effect of screening of negatively charged phosphate by the positive charge of the ring nitrogen. Since the calculated ΔT_m values were obtained for a dye which was assumed to have one charge in the molecule, the differences in the theoretical and experimental values probably reflect the influence of the second charge on the terminal nitrogen.

We were unable to find any effect of the charge on the acridine ring nitrogen on the biosynthesis of protein and RNA in cell culture. The charge has little effect on

Table 3

Increased stability of the secondary structure of DNA after complexing of acridine derivative

Buffer concentration, 0.01 M, pH 7.0. Each value of ΔT_m found and calculated represents the average from three independent experimental data. r is the number of dye molecules per one DNA nucleotide, and r', the number of dye molecules charged in the ring per one nucleotide.

Derivative*	r	r'	ΔT_m	
			calculated	found
C-337 (2)	0.04	0.004	0.3	0.6
C-283 (3)	0.05	0.045	3.0	3.5
C-609 (5)	0.06	0.06	4.0	4.7

*The number of carbon atoms in the side chain of each dye is indicated in parentheses.

complex formation between acridine derivatives and DNA but it has considerable influence on the ΔT_m of the complexed DNA. No direct correlation was observed between inhibition by the derivatives studied of RNA and protein synthesis, and the results of binding experiments, the melting temperatures of DNA-dye complexes, or the acid-base equilibrium of dyes. The cytostatic action of l-nitro-9-aminoacridine derivatives could be related to their ability to form a complex with DNA under physiological conditions. However, the existence of a positive charge on the acridine ring is not a prerequisite for the biological activity of the compounds studied. One cannot exclude, either, that other mechanisms than interaction with DNA might be responsible for cytostatic effects of the dyes studied (see e. g. Gumińska, 1973). Besides, it is not clear at the moment whether acridine derivatives undergo the metabolic conversion when put in contact with the living cell.

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KOMPLEKSY POCHODNYCH 1-NITRO-9-AMINOAKRYDYNY Z DNA

Streszczenie

Podstawione 1-nitro-9-aminoakrydyny hamują syntezę RNA i białka w hodowli tkankowej komórek ludzkich. Tworzenie się kompleksów badanych związków z DNA może być uważane za odpowiedzialne za ich działanie cytostatyczne. Istnieją dwa rodzaje kompleksów badanych pochodnych akrydyny z DNA, różniące się siłami wiążącymi. Aktywność biologiczna badanych związków nie zależy od obecności dodatniego ładunku w pierścieniu akrydynowym.

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SYNTHESIS AND PROPERTIES OF SOME 2-, 4- AND 5-AMINOPYRIMIDINES. EFFECT OF $-NH_2$ AND ORTHO-C METHYLATION ON PROTOLYTIC EQUILIBRIA AND ELECTRONIC ABSORPTION SPECTRA*

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A number of new 2-, 4- and 5-aminopyrimidines with sterical hindrance to the amino group rotation were synthesized. The pK_a values and u.v. absorption spectra of the aminopyrimidines were measured in order to elucidate the conformation of the amino group depending on the place of substitution.

Conformation and barrier to rotation of amino groups of pyrimidine and purine bases are of significance in understanding of some physico-chemical properties of these compounds and of the formation of double-helical complexes of respective polynucleotides. Alkylation of hydrogen bond donor group such as adenosylyl-N^6H_2 or cytidylyl-N^4H_2 (Van Holde *et al.*, 1965; Thedford & Straus, 1974; Griffin *et al.*, 1964; Brimacombe & Reese, 1966) might hinder the normal base pairing dependent on the orientation of the alkylamino group relative to the pairing bases. The presence of an alkyl substituent in ortho position relative to alkylamino group might further influence the conformation, hydrogen bonding and acid-base properties of aminopyrimidines and aminopurines.

So far anilines have been the group of compounds on which most work has been done with respect to the effects of sterical hindrance to amino group rotation on the physico-chemical properties of aromatic system (Jaffe & Orchin, 1962; Brown, H. C. *et al.*, 1955). In heteroaromatic system like pyrimidines the presence of two powerful electron-withdrawing ring nitrogens exerts a strong influence on the mesomeric interaction of electron-donating substituents, thus increasing the energy barrier to rotation of the amino group (Katritzky & Tiddy, 1969; Almog & Meyer, 1972). Systematic studies (Berens *et al.*, 1971; Berens, 1971; Smagowicz, 1974; Smagowicz

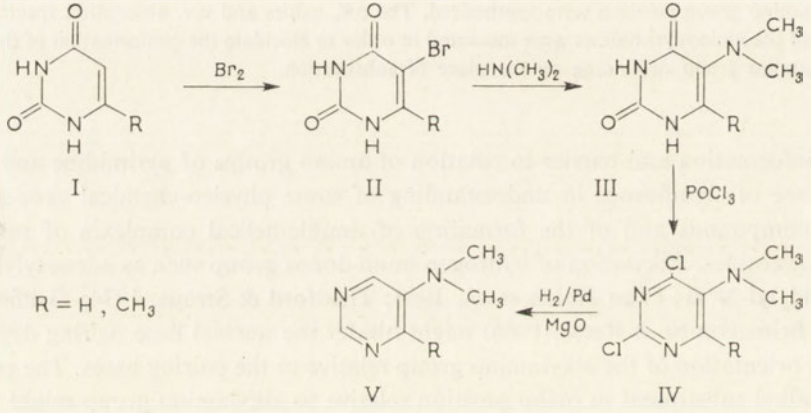
* This work was supported by the Polish Academy of Sciences within the project 09.3.1.

et al., 1974) on the effect of amino group substitution and its conformation on the excited states of pyrimidines carried out in this laboratory led us to synthesize several so far unknown dialkylamino derivatives with steric hindrance to rotation around the C-N bond, imposed by the presence of an ortho C-methyl group.

The synthesis, spectral and acid-base properties of aminopyrimidines including hindered and non-hindered derivatives were studied to elucidate the effect of the amino group conformation on the ground state properties of this group of compounds.

RESULTS AND DISCUSSION

Synthesis. At first an attempt was made at synthesizing a series of *N,N*-dialkylamino derivatives of 5-amino-4-methylpyrimidine using 5-bromo-4-methylpyrimidine as starting material (Van der Plas, 1965). However, according to this method only 5-amino-4-methylpyrimidine was obtained by amination with concentrated ammonia in the presence of copper dust which greatly facilitated formation of the final product. Dimethylamine or diisobutylamine made to react with 5-bromo-4-methylpyrimidine under various experimental conditions either proved to be non-reactive or gave a series of unidentified products most probably resulting from a cleavage reaction.



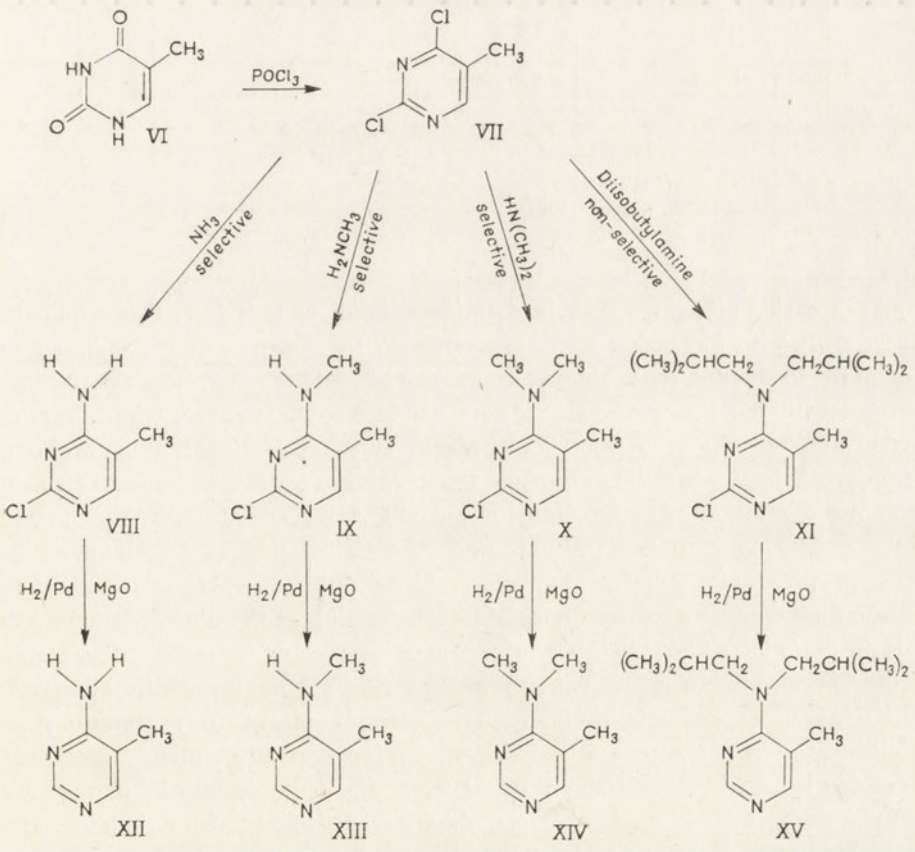
Scheme 1

Therefore, another route was devised (Scheme 1): 6-methyluracil (I, $R=CH_3$) was first brominated (Elderfield & Prasad, 1960) to II, $R=CH_3$, which in the presence of dimethylamine (Chernova & Khokhlov, 1960) gave III, $R=CH_3$. Subsequent chlorination (IV, $R=CH_3$) and reductive removal of chlorine atom by hydrogenation in the presence of Pd and MgO in ethanolic solution finally yielded 5-dimethylamino-4-methylpyrimidine (V, $R=CH_3$). In a similar way 5-dimethylaminopyrimidine (V, R=H) was obtained from uracil (I, R=H) by bromination (Wang, 1959), amination with dimethylamine and chlorination (O'Brien *et al.*, 1966), and reduction with hydrogen over Pd. Diisobutylamine, however, reacted

neither with 5-bromouracil nor 5-bromo-6-methyluracil; under more rigorous conditions only hydrolytic removal of bromine was observed.

In order to obtain a series of hindered 4-aminopyrimidines (Scheme 2) thymine (VI) was first converted to 2,4-dichloro-5-methylpyrimidine (VII) (Bhat & Munson, 1968) and then aminated by treatment with ammonia, methylamine, dimethylamine (Koppell *et al.*, 1962), or diisobutylamine. The reactions were very selective and yielded the respective 4-amino, alkylamino and dialkylamino derivatives (VIII - XI). Only in the case of diisobutylamine, in addition to the main product, small amounts of 2-diisobutylamino-4-chloropyrimidine and 2,4-bis-diisobutylamino-4-chloropyrimidine were formed. Reductive removal of chlorine atom from VIII - XI by hydrogenation over Pd in ethanolic solution in the presence of MgO gave 4-amino--5-methylpyrimidine (XII), 4-methylamino-5-methylpyrimidine (XIII), 4-dimethylamino-5-methylpyrimidine (XIV) and 4-diisobutylamino-5-methylpyrimidine (XV), respectively. 4-Dimethylaminopyrimidine (XVIII) devoid of an ortho methyl group was synthesized from uracil in the analogous way.

In the series of 2-aminopyrimidines, sterical hindrance to the amino group rotation could only be achieved by replacement of NH_2 hydrogens by bulky alkyl



Scheme 2

groups. 2-Diisobutylaminopyrimidine (XIX) was thus obtained by treatment of 2-chloropyrimidine with diisobutylamine.

Protolytic equilibria. Proton dissociation constants for the newly synthesized aminopyrimidines were determined spectrophotometrically (Table 1). It has been previously demonstrated (Brown, D. J. *et al.*, 1955) that the basic protonation centre in aminopyrimidines, irrespective of the position of amino group attachment, lies

Table 1

pK_a *values of the unhindered and hindered 2-, 4- and 5-aminopyrimidines*

Aminopyrimidine	pK_a	Aminopyrimidine	pK_a
5-NH_2-	2.60[a]	5-NH_2-4-CH_3-	3.15[c]
5-$N(CH_3)_2$-	3.23	5-$N(CH_3)_2$-4-CH_3-	2.86
4-NH_2-	5.71[b]	4-NH_2-5-CH_3-	6.06
4-$NHCH_3$-	6.12[d]	4-$NHCH_3$-5-CH_3-	6.73
4-$N(CH_3)_2$-	6.35[d]	4-$N(CH_3)_2$-5-CH_3-	6.64
		4-N(CH$_2$-CH(CH$_3$)CH$_3$)$_2$-5-CH_3-	6.34
2-NH_2-	3.54[b]		
2-$N(CH_3)_2$-	3.96[d]		
2-N(CH$_2$-CH(CH$_3$)CH$_3$)$_2$-	3.67		

[a] Whittaker (1951).
[b] Albert *et al.* (1948).
[c] Marshall & Walker (1951).
[d] Brown & Short (1953).

always on a ring nitrogen atom. In 4-aminopyrimidines it is N-1. One would thus expect that, with increasing steric hindrance to the rotation of the amino group around the C-N bond, the resonance interaction of the lone electron pair of the amino nitrogen with the heteroaromatic system would become weaker, with a resultant decrease in the basicity of the ring nitrogen. This expectation is fully borne out for 5-dimethylaminopyrimidine, pK_a of which exhibits a drop by about 0.4 pK unit upon introduction of an ortho-methyl group at C-4 carbon atom. This decrease is in fact rather close to one pK unit since C-4-methylation of 5-aminopyrimidine increases pK_a by 0.55 unit. This means that the donating effect of C-methylation is more than compensated by sterically forced rotation of the dimethylamino group from the pyrimidine plane. Such steric hindrance imposed on the resonance interaction of the dimethylamino group with pyrimidine ring cannot be readily observed in 4-dimethylamino-5-methylpyrimidine. The latter, contrary to expectation, is a stronger base than the unhindered dimethylamino derivative. Steric hindrance to resonance interaction may be presumed from the decrease in pK_a in the order methylamino- and dimethylamino- to diisobutylamino-5-methylpyrimidine. However, a similar decrease in pK_a is observed in 2-diisobutylaminopyrimidine, as com-

pared with pK_a of the dimethylamino analogue. We are thus tempted to assume that the presence of bulky alkyl groups may both sterically and hydrophobically influence solvation of that part of the molecule which is close to protonation centre and may, in turn, decrease its ability to accept a proton from an oxonium cation. A similar effect of *N,N*-alkyl substitution on the protonation ability of the amino nitrogen atom has been sought also in lowered basicity of dialkylamino anilines (Hall & Spinkle, 1932). In the light of the presented data the conformation of the amino group in 4-dimethylaminopyrimidines seems to be only slightly affected by ortho-methyl substitution. Much more pronounced changes in pK_a of the same protolytic reaction between 5-methyl- and 5-ethylcytosine derivatives are observed upon *N,N*-methylation (Kulikowski *et al.*, 1969; Kulikowski & Shugar, 1971). Replacement of one of the NH_2 hydrogen atoms by the CH_3 group in 1,5-dimethylcytosine causes a decrease in pK_a by 0.2 unit; in 1,N^4,N^4,5-tetramethylcytosine, pK_a exhibits an additional decrease by 0.1 unit. The corresponding changes in the pK_a of 5-ethylcytosine and 5-ethylcytidine brought about by monomethylation of the amino group are somewhat greater and they are in agreement with expectation based on stereochemical considerations. The conformation of the amino group in cytosines corresponds closely to its co-planarity with the ring plane (Shoup *et al.*, 1972); consequently, in the presence of a bulky *N*-methyl group in 5-methyl- or 5-ethylcytosines the amino group is forced to assume a less strained conformation by rotation from the molecular plane around the C-N bond. In 4-aminopyrimidines the equilibrium conformation of the amino group apparently deviates from co-planarity. Differences in this respect between cytosines and 4-aminopyrimidines are most probably due to the presence of amide carbonyl in the former group of compounds giving rise to larger contribution of mesomeric structures.

U. v. absorption spectra. The absorption spectra in ethanol solution of 4- and 5-aminopyrimidines sterically hindered by an ortho-methyl group are presented in Table 2 together with the redetermined absorption spectra of the parent non-hindered molecules, and 2-aminopyrimidines. The shapes of the absorption bands of unhindered and hindered compounds are similar. Therefore, instead of the integrated intensities, molar absorptivities were determined and used in the discussion of the steric effects. The spectra of all non-hindered amino- and dimethylaminopyrimidines are in good agreement with those obtained previously (Whittaker, 1951; Brown & Short, 1953). The spectrum of 5-dimethylaminopyrimidine is obtained for the first time by us. Of the two absorption bands seen in the near ultraviolet region of the spectrum the lowest energy band has been demonstrated (Berens *et al.*, 1971; Smagowicz *et al.*, 1974) to correspond to an $a_{\pi^*} \leftarrow 1$ electronic transition of a considerable intramolecular charge-transfer character, similar to that observed in anilines (Kasha & Rawls, 1968). Charge-transfer states associated with promotion of a lone-pair electron of the amino group to an antibonding π^*-orbital of the pyrimidine ring mix with locally excited π,π^* states and give rise to 1, a_{π^*} states. The degree of mixing and, in turn, the energy and oscillator strength of $a_{\pi^*} \leftarrow 1$ transitions, depend on the angle of the amino group twist around the essentially single C-N bond (Kasha & Rawls, 1968; Lim & Chakrabarti, 1967). When $\Theta = 0$, i. e. at maximum overlap

Table 2

U.v. absorption of the unhindered and hindered 2-, 4-, and 5-aminopyrimidines in ethanol

Aminopyrimidine	$\lambda_{1\,max}$ (nm)	$\lambda_{2\,max}$ (nm)	$\varepsilon_{1\,max}$	$\varepsilon_{2\,max}$
5-NH_2-	245	314	12 900	3100
5-$N(CH_3)_2$-	261	332	15 800	2700
4-NH_2-	234	272	20 600	5130
4-$N(CH_3)_2$-	249	285	16 900	3620
2-NH_2-	226	296	16 600	3890
2-N $(CH_3)_2$-	243	314	20 100	2370
2-N(CH_2-$CH(CH_3)_2$)-	248	319	20 600	2120
5-NH_2-4-CH_3-	244	307	10 500	3830
5-$N(CH_3)_2$-4-CH_3-	267	315	5 420	1670
4-NH_2-5-CH_3-	234	273	10 700	4850
4-$NHCH_3$-5-CH_3-	243	276	14 200	5080
4-$N(CH_3)_2$-5-CH_3-	259	290*	11 900	4890*
4-N(CH_2-$CH(CH_3)_2$)-5-CH_3-	262	298*	11 700	3960*

* Absorption maximum not clearly defined. Its position and extinction were evaluated after graphic subtraction assuming similar shapes of the two bands in the spectra of both compounds.

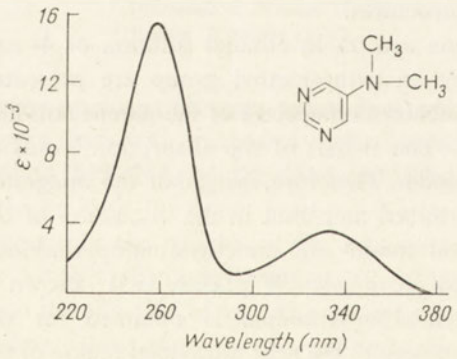


Fig. 1

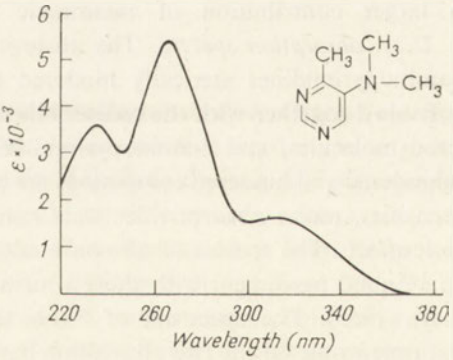


Fig. 2

Fig. 1. U.v. spectrum of 5-dimethylaminopyrimidine in ethanol.
Fig. 2. U.v. spectrum of 5-dimethylamino-4-methylpyrimidine in ethanol.

of the *p* orbital of the substituent and 2 *pπ* orbital of the adjacent carbon atom of the pyrimidine ring, the mixing attains a maximum value, while the $a_{\pi^*} \leftarrow 1$ transition exhibits the lowest energy and the highest oscillator strength. When this angle is 90°, there is no mixing between the locally excited and charge-transfer states, and the spectrum is expected to be composed of the absorption bands associated with local

excitations of the aromatic ring and the substituent. From the potential-energy diagrams of the angle of twist (Jaffe & Orchin, 1960) a hypochromic effect can be predicted in the case of slight steric interference by an ortho group while at moderate hindrance the appearance of both a hypsochromic and hypochromic effect is expected.

Inspection of the data contained in Table 2 shows that the steric effects in the spectra of aminopyrimidines do not manifest themselves as simply as it would follow from the above picture. First of all, on introduction of an ortho-methyl group into 5-aminopyrimidine both absorption bands λ_1 and λ_2 show a hypsochromic shift with concomitant reduction in intensity of the higher-energy band as if the 4-methyl group imposed moderate hindrance to rotation of the neighbouring amino group. However, at the same time the lower-energy band considered to correspond to the $a_{\pi^*} \leftarrow 1$ transition becomes more intense. In 5-dimethylamino-4-methylpyrimidine the intensity of both bands is reduced by about 50%, as compared with that of 5-amino-4-methylpyrimidine; contrary to expectation a bathochromic shift of the two bands is observed. Since introduction of the 4-methyl group into 5-dimethylaminopyrimidine brings about hypsochromic and hypochromic effects on the λ_2 band, the bathochromic effect in the former case can be attributed to the lower ionization energy of the dimethylamino group. It should be pointed out, however, that 4-methyl substitution shifts the λ_1 band in 5-dimethylaminopyrimidine bathochromically and decreases its intensity to 1/3. In 4-aminopyrimidines the effects of ortho-methyl substitution on the absorption spectra of unhindered derivatives are much less pronounced, and differ somewhat in character from that observed for 5-aminopyrimidines. The spectra of 4-aminopyrimidine and its 5-methyl derivative are similar, except that the intensity of the λ_1 band in the hindered derivatives is lower by about a half. Replacement of the amino group by the substituents of progressively larger size, i. e. methylamino, dimethylamino and diisobutylamino, is accompanied by only slight changes in the intensity of both absorption bands and by gradual lowering of their energy. Introduction of the 5-methyl group to 4-dimethylaminopyrimidine results in a bathochromic effect; the higher-energy band exhibits a much smaller decrease in intensity as compared with 5-dimethylaminopyrimidine. The longer wavelength component becomes at this same time more intense.

There is no doubt that explanation of the divergent effect of ortho-methyl substitution on the λ_1 and λ_2 bands of 4- and 5-aminopyrimidines would require theoretical studies and knowledge of the potential-energy diagrams for amino group rotation. Nevertheless on the basis of the presented data some qualitative conclusions can be drawn concerning the contribution of charge-transfer states to the observed transitions and conformations of the unhindered molecules. Since any substitution of an aromatic system with an electron-donating group lowers the energy of electronic transitions, and decreases the ionization potential of amino group by *N*,*N*-alkyl substitution (Kwiatkowski, 1966) on the $a_{\pi^*} \leftarrow 1$ transitions (Table 2), therefore it is difficult to distinguish contributions of these and hypsochromic steric effects in the spectra of hindered aminopyrimidines. Thus, considering only the hypochromic effects of ortho-methyl substitution it can be concluded that in 5-amino-

pyrimidine and 5-dimethylaminopyrimidine: 1) both transitions found in their absorption spectra exhibit marked charge-transfer character, 2) large hypochromic changes induced by the 4-methyl group indicate that the latter forces rotation of the amino (alkylamino) group from the molecular plane, in order to relieve conformational strain imposed by steric crowding. Introduction of the 5-methyl group in 4-aminopyrimidine apparently does not affect conformation of the molecule. Even conformation of 4-dimethylaminopyrimidine and 4-diisobutylaminopyrimidine bearing bulky alkyl substituents seem to be changed to a lesser extent than the corresponding ortho-methyl derivatives of 5-dimethylaminopyrimidine. Therefore, it can be concluded that in 4-aminopyrimidines the amino group attains a non-planar conformation. In all isomeric unhindered aminopyrimidines the replacement of the amino group by dimethylamino is accompanied by a large decrease in the intensity of the lowest energy $a_{\pi^*} \leftarrow 1$ transition. This can be taken as an indication of a more twisted conformation of the dimethylamino than amino group, though the space-filling scale models of these molecules do not show any hindrance to rotation. It is also noteworthy that these steric effects are hardly observed in the u. v. spectra of hindered 5-alkylcytosines, in apparent disagreement with the essentially planar conformation of their amino group.

Interpretation of the spectral properties of the cationic forms in terms of steric effects is difficult, since both absorption bands of the protonated 4-aminopyrimidines as well as the two shorter wavelength bands in the case of protonated 5-dimethylaminopyrimidine, are merged into one band. For this reason a descriptive comparison with the claimed steric effects in the spectra of the cationic species of 5-alkylcytosines (Kulikowski & Shugar, 1971) is hardly possible.

Further physico-chemical studies are needed to get better insight into the conformation of the amino group substituents in aminopyrimidines. Infrared and dielectric investigations are carried out in this laboratory and will be reported elsewhere.

EXPERIMENTAL

U. v. absorption measurements were performed using a Carry 118 recording spectrophotometer. Dissociation constants (pK_a) were determined spectrophotometrically on a Zeiss (Jena) VSU2P manual spectrophotometer, while pH was measured to an accuracy of 0.02 unit on a Radiometer PHM 22 pH-meter.

5-Bromo-6-methyluracil (II, $R = CH_3$) was prepared according to Elderfield & Prasad (1960).

5-Dimethylamino-6-methyluracil (III, $R = CH_3$) was obtained as described by Chernova & Khokhlov (1960).

5-Dimethylamino-2,4-dichloro-6-methylpyrimidine (IV, $R = CH_3$): 9 g of III, 20 ml of *N,N*-dimethylaniline and 250 ml of freshly distilled $POCl_3$ were refluxed for 4.5 h at 115°C. The excess of phosphorus oxychloride was then removed by evaporation and the syrupy residue was poured onto crushed ice. The aqueous solution was extracted three times with 250 ml portions of ether. The combined ether extract was washed with water and dried over anhydrous Na_2SO_4. After

evaporation of ether, the resulting oily residue was distilled under reduced pressure to yield 9.2 g of IV, R = CH_3 (84% yield), with b. p. 90-96°C at 1 mm Hg, $\lambda_{1\,max}^{ethanol}$ 261 nm and $\lambda_{2\,max}^{ethanol}$ 298 nm. (Found: C, 40.78; H, 4.25; N, 20.53; Cl, 34.33. $C_7H_9N_3Cl_2$ requires: C, 40.77; H, 4.40; N, 20.39; Cl, 34.40%).

5-Dimethylamino-4-methylpyrimidine (V, R = CH_3): A mixture of 2.8 g of IV, 150 ml of ethanol and 1 g of 10% Pd on charcoal was hydrogenated with H_2 in the presence of an excess of MgO under normal pressure at room temperature. The progress of the reaction was followed chromatographically using silica-gel plates and chloroform-methanol (9:1) as a solvent. After completion of the reduction the reaction mixture was filtered and the filtrate evaporated. The residue was dissolved in water and the solution extracted three times with 50 ml portions of chloroform. The extract was dried over anhydrous Na_2SO_4 and the solvent evaporated. The oily product was distilled under reduced pressure to yield 1.95 g of V, R = CH_3 (62%), b. p. 56 - 58°C at 1 mm Hg, $\lambda_{1\,max}^{ethanol}$ 267 nm, $\lambda_{2\,max}^{ethanol}$ 315 nm (Found: C, 61.19; H, 8.05; N, 30.57. $C_7H_{11}N_3$ requires: C, 61.26; H, 8.08; N, 30.64%).

5-Bromouracil (II, R = H) was obtained according to the procedure of Wang (1959).

5-Dimethylaminouracil (III, R = H) was obtained as described by O'Brien *et al.* (1966).

5-Dimethylamino-2,4-dichloropyrimidine (IV, R = H): 4 g of III, R = H, in 100 ml of $POCl_3$ and 8 ml of *N,N*-dimethylaniline were refluxed for 4 h. Then the excess of $POCl_3$ was evaporated and the remaining syrupy residue was poured onto crushed ice. The product was then extracted with ethyl ether and crystallized from heptane with final yield of 60%. M. p. 90°C, $\lambda_{max}^{ethanol}$ 277 nm.

5-Dimethylaminopyrimidine (V, R = H): 0.95 g of IV, R = H, in 50 ml of ethanol was hydrogenated as described for V, R = CH_3. The reaction was followed chromatographically using silica-gel plates and chloroform - methanol (9:1) as solvent. After complete reduction of chlorine the remaining slurry was filtered and the filtrate was evaporated and dissolved in water, and the product was extracted with chloroform. After crystallization from light petroleum, 0.26 g (45% yield) of V, R = H, was obtained. M. p. 78°C; $\lambda_{1\,max}^{ethanol}$ 261 nm, $\lambda_{2\,max}^{ethanol}$ 332 nm. (Found: C, 58.16; H, 7.53; N, 34.54. $C_6H_9N_3$ requires: C, 58.49; H, 7.37; N, 34.13%).

2,4-Dichloro-5-methylpyrimidine (VI) was obtained as described by Bhat & Munson (1968).

4-Dimethylamino-2-chloro-5-methylpyrimidine (X) was obtained according to Koppell *et al.* (1962).

4-Dimethylamino-5-methylpyrimidine (XIV): 1.5 g of X in 50 ml of ethanol was hydrogenated (with hydrogen gas) under normal pressure at room temperature, in the presence of 0.5 g of 10% Pd on charcoal and an excess of MgO. The progress of the reaction was followed by t. l. c. on silica-gel plates (chloroform - methanol, 9:1). When the reduction was completed the reaction mixture was filtered and evaporated; the residue was dissolved in water and three times extracted with chloroform. The chloroform solution was dried using Na_2SO_4, the solvent was evaporated and

XIV was distilled under reduced pressure to yield 0.49 g (40% yield) of colourless liquid, b.p. 68°C at 1 mm Hg. $\lambda_{max}^{ethanol}$ 259 nm. (Found: C, 61.19; H, 7.93; N, 30.63. $C_7H_{11}N_3$ requires: C, 61.26; H, 8.08; N, 30.64%).

4-Diisobutylamino-2-chloro-5-methylpyrimidine (XI): A solution of 2.1 g of VII and 4.5 ml of diisobutylamine in 20 ml of ethanol was stirred at room temperature for 10 h. The main product of the reaction (XVI) was separated by preparative t. l. c. on silica-gel plates using chloroform as solvent. The product (1.55 g, 47% yield) was crystallized from light petroleum. M.p. 57 - 58°C, $\lambda_{1\,max}^{ethanol}$ 263 nm, $\lambda_{2\,max}^{ethanol}$ 292 nm. (Found: C, 60.56; H 8.69; N, 16.53; Cl, 13.84. $C_{13}H_{22}N_3Cl$ requires: C, 61.02; H, 8.67; N, 16.43; Cl, 13.85%).

4-Diisobutylamino-5-methylpyrimidine (XV): 1.1 g of XI was reduced according to the procedure described for XIV, to yield 0.61 g (64%) of XV, after purification by distillation under reduced pressure, b.p. 90 - 92°C at 1 mm Hg, $\lambda_{max}^{ethanol}$ 262 nm. (Found: C, 70.75; H, 10.06; N, 19.22. $C_{13}H_{22}N_3$ requires: C, 70.52; H, 10.48; N, 18.99%).

4-Methylamino-2-chloro-5-methylpyrimidine (IX) was prepared as described by Koppell *et al.* (1962).

4-Methylamino-5-methylpyrimidine (XIII): Reduction with hydrogen gas, of 1.6 g of IX according to the procedure described for XIV and XV gave after crystallization from heptane 0.85 g (55% yield) of XIII. M. p. 123 - 124°C, $\lambda_{1\,max}^{ethanol}$ 243 nm and $\lambda_{2\,max}^{ethanol}$ 276 nm. (Found: C, 58.26; H, 7.34; N, 34.40. $C_6H_9N_3$ requires: C, 58.49; H, 7.37; N, 34.13%).

4-Amino-2-chloro-5-methylpyrimidine (VIII): 1.4 g of VII was added to 12 ml of 20% aqueous ammonia and the reaction mixture was stirred for 24 h. The solution was then evaporated to dryness. The product was crystallized (0.83 g, 72% yield) from anhydrous ethanol. M. p. 220°C, $\lambda_{1\,max}^{ethanol}$ 234 nm, $\lambda_{2\,max}^{ethanol}$ 276 nm. (Found: C, 41.28; H, 4.17; N, 29.20; Cl, 24.24. $C_5H_6N_3Cl$ requires: C, 41.80; H, 4.21; N, 29.27; Cl, 24.70%).

4-Amino-5-methylpyrimidine (XII): 0.58 g of VIII dissolved in 100 ml of ethanol was reduced with hydrogen gas under atmospheric pressure at room temperature, in the presence of 0.25 g Pd on charcoal and an excess of MgO. Observation of the progress of the reaction and separation of the product were achieved using t. l. c. on silica-gel plates and chloroform - methanol (85:15) as solvent. The final yield of XII was 0.213 g (48%) after crystallization from heptane. M. p. 176°C, $\lambda_{1\,max}^{ethanol}$ 235 nm, $\lambda_{2\,max}^{ethanol}$ 273 nm. (Found: C, 55.00; H, 6.46; N, 38.52. $C_5H_7N_3$ requires: C, 54.36; H, 6.49; N, 38.41%).

2,4-Dichloropyrimidine (XVI) was synthesized according to Bhat & Munson (1968).

4-Dimethylamino-2-chloropyrimidine (XVII): To 10 ml of an ethanolic solution of 4.2 g of XVI, 9.4 ml of a 33% ethanolic solution of dimethylamine were added dropwise with vigorous stirring. After 2 h the mixture was evaporated and the product purified chromatographically using silica-gel plates and chloroform as solvent. Crystallization from heptane gave 1.4 g of XVII (32% yield). M. p. 79°C, $\lambda_{1\,max}^{ethanol}$ 249 nm, $\lambda_{2\,max}^{ethanol}$ 288 nm.

4-Dimethylaminopyrimidine (XVIII): 0.7 g of compound XVII was hydrogenated with H_2 in the presence of 10% Pd on charcoal according to the procedure described for XIII, XIV and XV. The product was obtained in 53% yield (0.29 g), b.p. 70°C at 1 mm Hg, $\lambda_{1\,max}^{ethanol}$ 249 nm, $\lambda_{2\,max}^{ethanol}$ 285 nm.

2-Diisobutylaminopyrimidine (XIX): To 50 ml of ethanol 1.5 g of 2-chloropyrimidine and 6 ml of diisobutylamine were added and the mixture was heated under reflux for 10 h. Ethanol was then evaporated and the residue extracted three times with 150 ml portions of chloroform. Distillation of the extract under reduced pressure gave 0.75 g (27.6%) of colourless liquid, b.p. 84°C at 1 mm Hg, $\lambda_{1\,max}^{ethanol}$ 248 nm $\lambda_{2\,max}^{ethanol}$ 319 nm. (Found: C, 67.92; H, 9.92; N, 20.29. $C_{12}H_{21}N_3$ requires: C, 69.50; H, 10.21; N, 20.28%).

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SYNTEZA I WŁASNOŚCI NIEKTÓRYCH 2-, 4- i 5-AMINOPIRYMIDYN. WPŁYW METYLACJI GRUPY AMINOWEJ I ORTO-C METYLACJI NA RÓWNOWAGI PROTOLITYCZNE I ABSORPCYJNE WIDMA ELEKTRONOWE

Streszczenie

Opisano syntezy kilku nowych 2-, 4- i 5-aminopirymidyn z zawadą przestrzenną dla swobodnego obrotu grupy aminowej. Zmierzono i przedyskutowano wartości pK_a oraz widma absorpcji w ultrafiolecie całej grupy aminopirymidyn celem ustalenia konformacji grupy aminowej w zależności od miejsca jej podstawienia do pierścienia.

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EFFECT OF MODIFICATION ON PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF HAPTOGLOBIN

ACETYLATION, IODINATION AND NITRATION

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Human haptoglobin (Hp) type 2-1 was modified with *N*-acetylimidazole, iodine or tetranitromethane (TNM), and the ability of the obtained derivatives to form with haemoglobin (Hb) complexes with peroxidase activity, was estimated. At low reagent to protein molar ratios, 11 tyrosine residues were nitrated, 12 acetylated and 13 iodinated. The biological activity of NO_2-Hp and I-Hp amounted to 40% of the activity of native Hp whereas the activity of Ac-Hp, only to 16%. The derivatives modified at high ratios of *N*-acetylimidazole or iodine lost the ability to bind with Hb. Deacylation of tyrosines and partial liberation of acetylated ε-amino groups resulted in partial recovery of the activity. As demonstrated by polyacrylamide-gel electrophoresis, the modification of Hp with high excess of TNM or iodine induced polymer formation.

Specific chemical modification of protein is a very efficient tool for recognizing the participation of a particular amino acid residue in the biological and immunological activity of the protein. A good way to assess the biological role of a given amino acid residue, is to modify it in more than one manner. However, certain limitations should be taken into consideration as, in addition to the specific reaction, side-reactions can occur.

Haptoglobin (Hp)[1], an α_2- acid glycoprotein of serum, binds haemoglobin (Hb) stoichiometrically and irreversibly (Jayle, 1951). The complex can be considered to be

[1] Abbreviations used: Hp, haptoglobin; Hb, haemoglobin; TNM, tetranitromethane; Ac-Hp, haptoglobin with tyrosine residues acylated by *N*-acetylimidazole; DeAc-Hp, haptoglobin with acyl groups removed by hydroxylamine; NO_2-Hp, haptoglobin with tyrosine residues nitrated by tetranitromethane; NH_2-Hp, haptoglobin with nitro groups reduced by sodium hydrosulphite; I-Hp, iodinated haptoglobin; TNBS, 2,4,6-trinitrobenzene sulphonic acid; PCMB, *p*-chloromercuribenzoate.

a "true" peroxidase. In previous work from our laboratory (Dobryszycka *et al.*, 1969; Dobryszycka & Bec, 1971), certain aspects of the role of exposed tyrosine residues in formation of the active Hp-Hb complex, and in antigenic reactivity of Hp, were studied following modification of these residues by means of *N*-acetylimidazole. The present study was undertaken in an attempt to examine the role of the tyrosyl residues of Hp in the interaction with Hb by comparing their susceptibility to acetylation, iodination and nitration. Specificity of *N*-acetylimidazole, iodine and tetranitromethane (TNM) as protein-modifying reagents was found to differ which was expressed by modification of other amino acids and conformational changes of the protein molecule (Shifrin & Solis, 1972; Takeda *et al.*, 1971; Riordan *et al.*, 1965). The character of the group introduced on tyrosyl residues of Hp and the rate of particular modifications were also investigated. A preliminary report of this work has appeared (Dobryszycka & Bec, 1972).

MATERIALS AND METHODS

Reagents. *N*-Acetylimidazole was purchased from Sigma Chem. Co. (St. Louis, Mo., U. S. A.), tetranitromethane from Koch-Light Laboratories (Colnbrook, Bucks., England), 2,4,6-trinitrobenzene sulphonic acid from Eastman Organic Chem. (Rochester, N. Y., U. S. A.); Sephadex G-25 was a Pharmacia (Uppsala, Sweden) product. All other chemicals from P. O. Ch. (Poland) were reagent grade quality. Human Hp_{2-1} was isolated from ascitic fluid as described by Dobryszycka & Lisowska (1966). Haemoglobin was prepared from horse erythrocytes by the method of McQuarrie & Beniams (1954).

Acetylation and deacylation. Acetylation with *N*-acetylimidazole was carried out essentially according to the procedure of Riordan *et al.* (1965). For each experiment 30 mg of Hp was dissolved in 3 ml of 0.01 M-Tris-HCl buffer, pH 7.5, and the indicated amount of the reagent was added. After 1 h the modified protein was separated from the unreacted reagent on a Sephadex G-25 column, and lyophilized. The number of modified tyrosyl residues was estimated from the decrease in absorbance at 278 nm using for calculations a molar differential extinction coefficient of 1160 obtained on *O*-acetylation of *N*-acetyltyrosine.

Deacylation of *N*,*O*-diacetyltyrosine derivatives of Hp was performed as described by Simpson *et al.* (1963) by incubation of 10 mg of Ac-Hp dissolved in 2 ml of 0.01 M--Tris-HCl buffer, pH 7.5, with 1 mM-hydroxylamine hydrochloride for 2 h at room temperature. Conditions of deacylation were carefully checked, shorter time or weaker concentration of the reagent being less efficient whereas longer time or higher concentration resulted in a complete loss of the peroxidase activity of the Hp-Hb complex. The reagent was separated by filtration through Sephadex G-25 column and the deacylated protein was lyophilized. The number of acetyl groups removed from Ac-Hp by hydroxylamine was calculated as follows: at 278 nm the molar absorption coefficient of 70 for *N*,*O*-diacetyltyrosine rises to 1230 on conversion to *N*-acetyltyrosine. This corresponds to the differential extinction coefficient of 1160 per mole of *N*,*O*-diacetyltyrosine deacylated.

Iodination. Iodination was carried out for 5 min in an ice bath in 0.02 M-glycine--NaOH buffer, pH 8.6, as described by Fattoum *et al.* (1971), using 0.01 M-iodine dissolved in 0.1 M-KI, at 5 - 100 molar ratio. The reaction was stopped by adding a sufficient amount of 0.1 M-sodium thiosulphate to react with any unreacted iodine, and the mixture was filtered through a Sephadex G-25 column equilibrated with 0.2 M-glycine - NaOH buffer, pH 8.6, at 4°C, then the protein was lyophilized. The content of iodotyrosines was determined from the changes in absorbance with pH, for monoiodotyrosine at 305 nm (pH 7.1 - 9.15), for diiodotyrosine at 325 nm (pH 5 - 7.1) and for tyrosine at 295 nm (pH 9.15 - 13) taking the ε values of, respectively, 4000, 4100 and 2400 (Wolf & Covelli, 1966).

Nitration and reduction of nitrotyrosyl residues. Nitration was carried out according to Riordan *et al.* (1967). For each experiment 20 mg of Hp was dissolved in 2 ml of 0.05 M-Tris - 1 M-NaCl buffer, pH 8.0, at room temperature, and TNM in ethanol at 5 - 1000 molar ratio, was added. After 1 h the mixture was passed through a Sephadex G-25 column equilibrated with 0.05 M-Tris - 1 M-NaCl buffer, pH 8.0, to remove nitroform and unreacted reagent. The absorbance of nitroprotein was measured at 428 nm, and for calculation of nitrotyrosyl residues the ε value of 4200 (Yamagami *et al.*, 1968) was taken.

Reduction of NO_2 groups into NH_2 was carried out according to Sokolovsky *et al.* (1967) by adding solid sodium hydrosulphite (about 40 moles per mole of NO_2 incorporated) in 0.5 M-Tris buffer, pH 8.0, containing 1 M-NaCl. The reaction mixture was stirred magnetically at room temperature for 30 min and then dialysed against water and lyophilized. The reduction of NO_2 groups was checked by the disappearance of the absorption at 428 nm, and the number of aminotyrosine residues was calculated using the ε value of 1600 at 275 nm and pH 3.0, or 4200 at 302 nm and pH 10.0 (Sokolovsky *et al.*, 1967).

Acetylation, nitration and iodination were also carried out with Hp submitted to 1 h action of 8 M-urea.

Analytical methods. Haptoglobin was assayed by the peroxidase method of Jayle (1951).

The free amino groups of native and lyophilized Ac-Hp preparation were determined according to Habeeb (1966). The protein, 1 mg/ml of 10% sodium dodecyl sulphate solution containing 4% NaOH (pH 8.5), was treated with TNBS (1 mg/ml) for 2 h at 40°C, and the number of amino groups reactive with TNBS was calculated from the absorbance at 335 nm and the molar extinction coefficient of 1.15×10^4 (Goldfarb, 1966). The decrease in the number of amino groups in Ac-Hp was taken as corresponding to the number of acylated amino groups.

Sulphydryl groups were determined with PCMB (Boyer, 1954) in 0.1 M-phosphate buffer, pH 7.0, using $\varepsilon = 7600$ at 255 nm.

Disc-gel electrophoresis in 7.5% polyacrylamide gel was performed according to Makonkawkeyoon & Haque (1970) with the glycine-Tris buffer system which gives a running pH of about 8.4. Electrophoresis was run at a constant current of 4 mA per tube for 1 h. The gels were then stained with 0.5% Amido Black 10 B in 7% acetic acid.

RESULTS

Acetylation and deacylation of haptoglobin. Treatment of haptoglobin with increasing amounts of *N*-acetylimidazole resulted in gradual incorporation of acetyl groups into tyrosyl residues (Table 1). The concomitant decrease in peroxidase activity of the acetylated Hp complexed with Hb, was related to the number of acetylated tyrosines and the reagent/protein molar ratio. At 120 molar excess of *N*-acetylimidazole, with 12 tyrosine residues modified, peroxidase activity of the complex with Hb was found to be 16%. At 400 molar ratio, the activity was completely abolished.

Table 1

Effect of N-*acetylimidazole concentration on the number of acetylated tyrosine residues in haptoglobin, and peroxidase activity of the Hp-Hb complex*

Mean values of 2 - 4 determinations are given. For Hp, the mol. wt. of 85 000 was taken for calculations. The peroxidase activity is expressed as percentage of the activity of the Hb complex with native Hp.

	N-Acetylimidazole to Hp molar ratio	Tyrosine residues acetylated per mole of Hp	Peroxidase activity (%)
	20	1.8	75.4
	40	4.1	64.4
	60	8.2	50
	80	11.2	20
	100	11.2	18
	120	12.0	16
	200	11.8	10
	250	11.8	6
	350	11.8	2
	400	11.8	0
	500	12.0	0
	1000	12.0	0
	1200	12.0	0
Urea-treated Hp	300	27.00	0

Acylation of tyrosine residues of Hp was accompanied by acylation of amino groups of the protein (Table 2). On deacylation with 1 mM-hydroxylamine "free" tyrosine residues were completely renatured while a few amino groups still remained acylated. The deacylation resulted in a significant rise in peroxidase activity even in the case when the acylated preparation showed no activity.

Iodination. Iodination of tyrosine residues of Hp and the concomitant loss of the Hb-binding capacity, exhibited some minor differences when compared with acetylation. At 11.2 tyrosyl residues acetylated, the Hp-Hb complex showed 20% of the original biological activity; at 10 tyrosyl residues iodinated, the preparation retained 40% of the activity (Table 3). At a high excess of *N*-acetylimidazole not more

Table 2

Effect of acetylation of amino groups in haptoglobin on the peroxidase activity of Hp-Hb complex

In acetylated and deacetylated Hp preparations, the amino groups and tyrosine were determined as described in Methods. Other details as in Table 1.

N-Acetylimidazole to Hp molar ratio	Acetylated amino groups in		Acetylated tyrosines in		Peroxidase activity (%)	
	Ac-Hp	DeAc-Hp	Ac-Hp	DeAc-Hp	Ac-Hp	DeAc-Hp
40	0					
60	2.9	2.9	8.2	0	50	70
80	7.3					
100	9.3	8.5	11.2	1	18	56
120	12.4	9.5				
1000	24.5		12.0	1	0	26
1200	37.7	8.5	12.0	0	0	21

than 12 tyrosyl residues were acetylated, whereas the high excess of iodine resulted in formation of 13 iodotyrosines. As a rule, monoiodotyrosine predominated but 2 - 2.5 diiodotyrosines were formed at higher iodine/Hp ratios.

Nitration and reduction of nitrotyrosyl residues. Successive nitration of the first 8 accessible tyrosine residues by the increasing excess of TNM, with concomitant decrease in peroxidase activity, resembled the processes observed on acetylation or iodination. When 11 tyrosyl residues were nitrated, Hp still retained 40% of its biological activity, a percentage exactly the same as in the case of iodination. Sam-

Table 3

Characteristics of iodo-derivatives of haptoglobin

Mean values of 2 - 4 determinations are given. For Hp, the mol. wt. of 85 000 was taken for calculations. Peroxidase activity is expressed as percentage of the activity of Hb complex with native Hp. For details see Methods.

	Iodine to Hp molar ratio	Tyrosine residues iodinated per mole of Hp	Monoiodotyrosine	Diiodotyrosine	Peroxidase activity (%)
	5	2.9	2.2	0.2	78
	10	3.9	3.6	0.9	73.5
	15	6.3	4.8	1.4	53
	20	9.1	8.8	1.4	44.5
	25	10.0	10.6	1.7	40.5
	30	12.7	11.1	2.4	39.5
	35	11.8	10.7	2.4	38
	50	12.8	12,4	2.1	12
	100	12.8	12.0	1.6	0
Urea-treated Hp	100	29.0	17.2	12.8	0

ples of Hp nitrated at high excess of TNM, which retained 30% of Hb-binding capacity, gave unexpectedly high values of nitrated tyrosine (Table 4). It should be noted that in Hp nitrated at 400 - 1000-fold excess of TNM, the determination of nitrotyrosine at 420 nm may be incorrect. Teuwissen *et al.* (1973) reported that some tyrosine residues could become destroyed and not to be found in the protein hydrolysate; simultaneously, spectral evidence pointed to the presence of nitrotryptophan. Therefore, the number of tyrosine residues nitrated by TNM should be estimated by tyrosine recovery in the hydrolysate.

Table 4

Nitration and reduction of nitrotyrosyl residues in haptoglobin

Mean values of 2 - 4 determinations are given. For Hp, the mol.wt. of 85 000 was taken for calculations. Peroxidase activity is expressed as percentage of the activity of Hb complex with native Hp. For details see Methods.

	TNM to Hp molar ratio	Tyrosine residues nitrated per mole of Hp	Peroxidase activity (%)	Nitrotyrosyl residues reduced	Peroxidase activity (%)
	5	0.8	82		
	10	3.3	79	2.7	70.0
	25	3.9	68		
	50	8.1	45		
	100	9.9	43	9.7	32.4
	150	10.0	41		
	200	10.8	40		
	400	15.0	30	15.5	22.5
	1000	17.0	29	19.5	17.5
Urea-treated Hp	300	27.0	0		

Reduction of nitrotyrosyl residues occurred quantitatively and resulted in a loss of additional 10% of the initial haptoglobin activity.

To determine any changes that might result from the effect of hydrosulphite alone, controls were prepared in which native Hp was treated with hydrosulphite under conditions similar to those employed in the reduction of NO_2-Hp, then the mixture was filtered through Sephadex G-25 and lyophilized. This Hp preparation was found to retain 91% of the original peroxidase activity when complexed with Hb, in spite of the evident reduction of disulphide bonds. On the other hand, after reduction of nitro derivatives of Hp, determination of SH groups showed only 1 SH group (Table 5).

Electrophoresis of modified haptoglobin preparations. The derivatives of Hp_{2-1} with tyrosyl residues modified at moderate excesses of the reagents, gave on polyacrylamide-gel electrophoresis similar patterns as the native Hp (Fig. 1, gels *2*, *4*, *8*); on the other hand, the samples modified at high excesses of the reagents used (gels *3*, *6*, *9*) differed in the number and mobility of bands. Deacylation of the sample

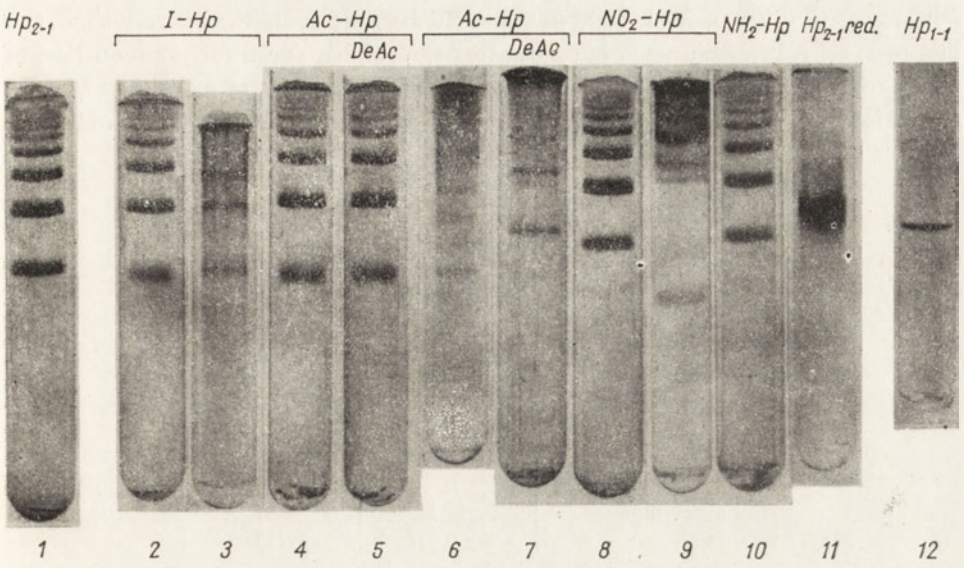

Fig. 1. Polyacrylamide-gel electrophoretic patterns of haptoglobin type 2 - 1 after iodination, acetylation or nitration. Characteristics of the preparations applied to the gels are given below.

Preparation	Tyrosyl residues modified	Reagent ratio	Peroxidase activity (%)	
1 Native Hp_{2-1}			(100)	
2 I-Hp	10	25	40	
3 I-Hp	13	100	0	
4 Ac-Hp	11	100	18	
5 DeAc-Hp	0		56	prep. no. *4*, deacylated
6 Ac-Hp	12	1000	0	25 NH_2 groups acetylated
7 DeAc-Hp	1		26	prep. no. *6*, deacylated, 16 NH_2 groups still acetylated
8 NO_2-Hp	10	100	43	
9 NO_2-Hp	17	1000	30	
10 NH_2-Hp	10		32	prep. no. *8*, reduced
11 Hp_{2-1} treated with hydrosulphite			91	
12 Hp_{1-1}			100	

treated with a 100-fold excess of *N*-acetylimidazole, although accompanied by a significant recovery of Hb-binding ability, did not result in parallel restoration of the characteristic electrophoretic pattern of Hp_{2-1} (gel *7*). High I_2/Hp ratio resulted in a decreased number of bands, moreover, a part of I-Hp did not enter into the gel, pointing to aggregation or polymerization (gel *3*). Electrophoresis of the derivative treated with a 100-fold molar excess of TNM (gel *9*) showed at least 10 bands, two of them migrating faster toward anode than the bands of Hp_{2-1}. A very interesting pattern was obtained with native Hp treated with hydrosulphite (gel *11*): after cleavage of disulphide bonds, there appeared one broad band accompanied by only

faint traces of other bands present in untreated Hp_{2-1}; the pattern is very similar to that of Hp_{1-1}. It should be pointed out that thiosulphate used for the reduction of nitrotyrosyl groups of Hp did not cause either an appearance of more than one SH group in these derivatives (see Table 5), or any changes in their electrophoretic patterns (gel *10*).

Table 5

Sulphydryl groups in hydrosulphite-treated haptoglobin

NH_2-Hp was obtained by reduction of NO_2-Hp containing 10 nitro groups; for reduction 40 moles of hydrosulphite was used. Native Hp was treated with the same amount of hydrosulphite. For details see Methods and Table 1.

Sample	Number of SH-groups per mole of Hp	Peroxidase activity (%)
Native Hp_{2-1}	0	100
Hp + hydrosulphite	12.3	91
NH_2-Hp	0.9	32

DISCUSSION

N-Acetylimidazole has been shown by Riordan *et al.* (1965) to be a rather selective reagent which preferentially acylates exposed tyrosyl groups, although it reacts also with amino and -SH groups. As Hp contains no free -SH groups, the latter reaction can be excluded but the extent of acetylation of the ε-amino groups has been found to increase at higher excess of the reagent. At 120 molar excess of *N*-acetylimidazole, when all the 12 accessible tyrosyl residues and 12 amino groups of Hp had been acetylated, peroxidase activity of the complex with Hb still amounted to 16%. At a higher molar excess, the acylation concerned only further amino groups and abolished peroxidase activity. Deacylation with hydroxylamine was followed by considerable restoration of peroxidase activity, but the obtained values never equalled those of native Hp. As under conditions of the experiment 11 - 12 tyrosyl residues were deacylated, the but partial recovery of the activity could be caused either by the incomplete deacylation of amino groups and/or by conformational changes visualized in polyacrylamide-gel electrophoresis (Fig. 1, gel *7*). In our opinion, a role of amino groups in biological activity of Hp may be excluded: when all of the accessible amino groups of Hp were blocked by azlactone of *p*-nitrobenzoylvaline, the obtained derivative retained full activity in the interaction with Hb (Dobryszycka & Osada, 1974); when succinic anhydride, a reagent causing an unfolding of the compact conformation of protein and a large increase of net negative charge, was used for the modification of amino groups in Hp, the peroxidase activity was reduced by one third (Dobryszycka & Osada, 1973). This seems to suggest that amino groups do not contribute to the formation of the active Hp-Hb complex, thus the loss of biological activity seems to be due solely to conformational alterations introduced by *N*-acetylimidazole.

Iodination of tyrosyl residues in protein may be accompanied by formation of a small but still significant amount of iodohistidines, even at low levels of iodination

(Seon *et al.*, 1971), and at higher concentration of iodine, also by modification of tryptophanyl residues (Denton & Ebner, 1971). The latter authors reported moreover polymer formation on iodination of α-lactalbumin with an at least tenfold excess of iodine. On the other hand, Shifrin & Solis (1972) on iodination of asparaginase observed no polymerization or dissociation into subunits. The results of our experiments seem to indicate that Hp iodinated at I_2/Hp ratio of 25:1 gave an unchanged electrophoretic pattern but at the 100:1 ratio aggregation and/or polymerization occurred (see Fig. 1, gels *2*, *3*). Iodination at high molar excess of the reagent resulted in similar changes in Hb-binding capacity as in the case of Hp submitted to a high excess of *N*-acetylimidazole. The decrease in enzymatic activity of particular iodo--derivatives of Hp in complexes with Hb could have been ascribed also to some blocking of histydyl or tryptophanyl residues. Chiao & Bezkorovainy (1972) have shown that histidines in Hp do not participate in Hb-binding whereas tryptophanyl residues are involved in this reaction (Dobryszycka *et al.*, 1969; Dobryszycka & Bec, 1971).

The appearance of diiodotyrosine was observed at I_2/Hp ratio of 10:1, and 2.4 diiodotyrosines per mole of Hp were formed when this ratio was 30:1. These data are fairly similar to those reported by Denton & Ebner (1971) for iodination of α-lactalbumin. Perlman & Edelhoch (1967) proposed that "the partly non-aqueous environment of "buried" tyrosyl residues directs their preferential conversion to diiodotyrosyl residues". If this is the case, then about 2 tyrosyl residues in Hp are relatively less exposed than the others.

Protein nitration by TNM is accompanied, in addition to specific modification of tyrosine, by numerous side-reactions, namely oxidation of sulphydryl groups, modification of tryptophan, methionine and histidine residues as well as formation of intermolecular, covalent cross-linked polymers (Denton & Ebner, 1971; Doyle *et al.*, 1968; Vincent *et al.*, 1970; Sokolovsky *et al.*, 1969). The results of Liu & Handschumacher (1972) indicate that the cross-linkage as a secondary effect of nitration with TNM is particularly enhanced in oligomeric enzymes.

Gradual nitration of Hp with increasing TNM/Hp ratio revealed 11 accessible tyrosyl residues in comparison with 13 in I-Hp, and 12 in Ac-Hp. It seems reasonable to assume that the tyrosines that react with iodine and not with TNM are less exposed (especially the one not reacting with *N*-acetylimidazole), and that the greater reactivity of iodine is due to its capacity to penetrate the hydrophobic surface of the protein because of its low polarity. The modification of 11 tyrosyl residues irrespective of the reagent used, indicates that they do not differ in reactivity toward the three reagents. However, differences are to be found in the biological activity of Hp derivatives. Hp treated even with a 1000-fold molar excess of TNM still retained 30% of its ability to activate Hb in spite of conformational changes (polymerization ?), whereas the corresponding Ac-Hp or I-Hp preparations were inactive.

The reduction of nitrotyrosyl residues in protein with sodium hydrosulphite leads largely to formation of the 3-aminotyrosyl derivatives with properties distinct from those of native protein, pK' values of 4.75 and 10.0 corresponding to the amino and hydroxyl groups, respectively (Sokolovsky *et al.*, 1967). The gradual loss of enzymic activity of Hp upon nitration was not reversed by reduction of the nitrotyro-

syl residues and even an about 10% further decrease of the activity was observed. Thus, the loss is most likely due to conformational changes induced by nitration of tyrosines. It should be noted that sodium hydrosulphite reduced nitrotyrosyl groups in Hp derivatives without cleaving disulphide bonds, although when native Hp was treated with this reagent 12 SH-groups appeared. Sodium hydrosulphite-treated Hp_{2-1} retained 91% of Hb-binding capacity but lost its polymorphic character and migrated in polyacrylamide gel as a single band with the mobility of Hp_{1-1}. It is noteworthy that Malchy *et al.* (1973) obtained a very similar electrophoretic pattern for Hp_{2-2} treated with sodium sulphite and PCMB.

Tyrosine reactivity in Hp was markedly increased when the modifications were carried out in 8 M-urea solution, showing that the different tyrosine reactivity observed in the native protein was the result of conformational determinants. Chiao & Bezkorovainy (1972) nitrated Hp_{2-1} with TNM obtaining 34 groups nitrated, while we found only 27 groups. The significance of this incomplete nitration at high TNM ratio is unclear. The results of Malan & Edelhoch (1970) indicate that intermolecular cross-linkages were formed when thyroglobulin or serum albumin were nitrated with large excess of TNM in 8 M-urea but were not formed when the native protein was nitrated in water. A similar mechanism may account for the above-mentioned results of ours.

The results of the present work point to the involvement of tyrosine residues in haptoglobin action on haemoglobin, the conformational changes induced either by acetylation, iodination or nitration influencing to a significant extent the biological activity of haptoglobin.

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WPŁYW MODYFIKACJI NA FIZYKO-CHEMICZNE I BIOLOGICZNE WŁAŚCIWOŚCI HAPTOGLOBINY

ACETYLOWANIE, NITROWANIE I JODOWANIE

Streszczenie

Ludzką haptoglobinę (Hp) typu 2-1 modyfikowano *N*-acetyloimidazolem, jodem i tetranitrometanem (TNM), po czym aktywność biologiczną określano na podstawie tworzenia kompleksu z hemoglobiną. Przy niskich stężeniach odczynników 11 reszt tyrozynowych ulega nitrowaniu, 12 acetylowaniu a 13 jodowaniu. Biologiczna aktywność pochodnych nitrowanych lub jodowanych wynosiła 40% aktywności natywnej Hp, a acetylowanych tylko 16%. Usunięcie grup acetylowych z tyrozyn i częściowe uwolnienie grup ε-aminowych powodowało częściową regenerację aktywności. Modyfikacja wysokimi stężeniami *N*-acetyloimidazolu lub jodu powodowała utratę zdolności wiązania hemoglobiny. Wykazano przy pomocy elektroforezy w żelu poliakrylamidowym, że wysokie stężenia TNM lub jodu powodowały tworzenie się polimerów.

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TYROSINE AMINOTRANSFERASE IN FROG LIVER

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1. In frog liver, tyrosine aminotransferase is located mainly in cytoplasm. The enzyme is an anionic protein of mol. wt. 115 000 daltons, specific toward 2-oxoglutarate. The enzyme separates on ion-exchange chromatography into two active forms.

2. Administration of triiodotyronine *in vivo* induces the activity of the enzyme. Epinephrine and glucagon have no effect, and cAMP and insulin repress this activity by about 70%.

3. Triiodotyronine stimulates incorporation of [^{14}C]leucine into protein, and the amount of the enzyme in the nascent polysome-bound protein is considerably increased.

Biosynthesis of tyrosine aminotransferase (L-tyrosine : 2-oxoglutarate aminotransferase, EC 2.6.1.5) is induced in mammalian liver by hydrocortisone (Lin & Knox, 1957; Kenney, 1962), insulin (Holten & Kenney, 1967), glucagon (Holten & Kenney, 1967; Wicks *et al.*, 1969), epinephrine and cyclic AMP (Wicks *et al.*, 1969). The induction concerns only the anionic form of the cytoplasmic enzyme. The cationic form of the enzyme present in mitochondria is not inducible by hormones (Miller & Litwack, 1969; Mertvetsov *et al.*, 1973).

The effect of these hormones on tyrosine aminotransferase in the frog has not been studied, with the exception of hydrocortisone which, as demonstrated by Michałek-Moricca (1965), did not stimulate the activity of this enzyme. The aim of the present work was to study the influence of other hormones on tyrosine aminotransferase in the frog. A preliminary report has been presented (Laskowska-Klita & Grójec, 1973).

MATERIAL AND METHODS

Animals. Adult frogs (*Rana esculenta*) weighing about 40 g and male albino rats weighing about 150 g, after overnight fasting, were used. In experiments on the effect of hormones, the animals were killed at determined time intervals after the injection, and the livers were isolated and rinsed with the solution used for homogenization.

Tissue extract. Livers were homogenized in 3 vol. of 0.14 M-KCl in a Waring blendor. The homogenate was centrifuged at 4°C at 30 000 *g* for 30 min and the supernatant (extract) used for determinations.

Mitochondrial fraction. Livers were homogenized with 9 vol. of 0.25 M-sucrose in a Potter-type glass homogenizer. The nuclei and cell debris were removed by centrifugation at 700 *g*; the mitochondria were sedimented at 14 000 *g* for 10 min at 4°C, suspended in 5 vol. per gram tissue of 50 mM-K-phosphate buffer, pH 7.2, supplemented with 1 mM-PLP[1] and 0.1 mM-2-oxoglutarate, and frozen at -20°C for 40 h, then homogenized in a glass homogenizer, centrifuged at 14 000 *g* for 10 min (Miller & Litwack, 1971), and the supernatant used for determinations.

Incorporation of [^{14}C]*leucine into liver slices.* This was carried out according to Auricchio *et al.* (1972). Slices 1 - 2 mm thick, 50 mg each, were prepared by hand; eight slices were incubated at 37°C in 5 ml of Krebs-Ringer phosphate buffer, pH 7.4 (Cohen, 1957) containing [^{14}C]leucine (5 μCi/ml). At 1 h intervals, two slices were withdrawn, washed twice with Krebs-Ringer buffer, pH 7.4, and homogenized by hand in 5 ml of 0.14 M-KCl containing 1 mM-PLP, 1 mM-EDTA and 1 mM-2-mercaptoethanol. A part of the homogenate was taken for determination of protein and enzyme activity; to the remaining part the same volume of 5% trichloroacetic acid (TCA) was added. After 30 min at 4°C, the mixture was centrifuged for 15 min at 2000 rev./min, the sediment washed three times with cold 5% TCA and dissolved in 1 ml of 1 M-NaOH. Radioactivity was measured in 50 μl samples in liquid scintillator (0.5% PPO and 0.015% POPOP in toluene), in an Isocap 300 scintillation counter (Nuclear-Chicago, U. S. A.), with an efficiency of 80%.

Polysome-bound tyrosine aminotransferase. Polysomes from liver of triiodothyronine-treated and untreated frogs were prepared and labelled according to Earl & Morgan (1968). The polysomes were isolated by discontinuous sucrose-gradient centrifugation and labelled for 5 min with [^{14}C]leucine in a cell-free system from liver of untreated frog. The labelled polysomes were isolated by centrifugation in 1 M-sucrose for 1 h at 280 000 *g*. The protein bound to polysomes was released by incubation with puromycin according to Earl & Hindley (1971) for 30 min at 0°C, then the mixture was chromatographed on CM-Sephadex column, 1.2 × 16 cm (see below). In the collected fractions, radioactivity and aminotransferase activity were determined.

Column chromatography. The DE-11 cellulose column (1.8 × 20 cm) and CM-Sephadex column (2 × 32 cm) were equilibrated with 50 mM-K-phosphate buffer, pH 6.8, containing 1 mM-EDTA, 1 mM-2-mercaptoethanol and 2 mM-2-oxoglutarate, and eluted with the same buffer followed by a linear 0 - 0.4 M-KCl or NaCl gradient in the same buffer.

Molecular weight of partially purified tyrosine aminotransferase was determined by gel filtration on Sephadex G-200 according to Andrews (1964). The enzyme was purified after Rosenberg & Litwack (1970) by ammonium sulphate fractionation

[1] Abbreviations used: pHPP, *p*-hydroxyphenylpyruvate; DTT, dithiothreitol; PLP, pyridoxal-5-phosphate.

(precipitate at 0.3 - 0.7 sat.), heating at 55°C and DE-11 cellulose chromatography. The purification was 200-fold and recovery 40% (unpublished).

Tyrosine aminotransferase activity was determined according to Diamondstone (1966). The enzyme, 0.8 - 1.4 mg protein, was preincubated for 5 min at 37°C with 19.2 μM-tyrosine, 0.16 μM-PLP and 0.2 M-Na,K-phosphate buffer, pH 8.2. The reaction was started by adding 2-oxoglutarate to a final concentration of 32 μM; final volume 3.2 ml. After 30 min incubation, 0.2 ml of 10 M-NaOH was added, and the extinction at 331 nm determined. To the control sample, NaOH was added prior to the addition of 2-oxoglutarate. The enzymic reaction was linear up to 60 min and the results are expressed as nmoles per minute, taking for calculations $\varepsilon = 19\,000$.

Protein was determined according to Lowry *et al.* (1951).

Chemicals. L-Tyrosine, epinephrine, and insulin were from Polfa (Warszawa, Poland), 2-oxoglutaric, pyruvic and oxaloacetic acids, and pyridoxal-5-phosphate from Fluka AG (Buchs SG, Switzerland), triiodotyronine from VEB Berlin-Chemie (Berlin-Adershof, G. D. R.), adenosine 3′,5′-phosphate, cyclic, from Serva (Heidelberg, G. F. R.), and glucagon from Eli Lilly (Indianapolis, Ind., U. S. A.). L-[U-^{14}C] Leucine (spec. act. 0.1 mCi/0.105 mg) was from the Institute of Radioisotopes (Prague, Czechoslovakia), 2,5-diphenyloxazole (PPO), 1,4-di-2(5-phenyloxazolyl)benzene (POPOP), cytochrome *c* from horse heart and rabbit γ-globulin, fraction II, were from Koch-Light Lab. (Colnbrook, Bucks, England), ATP, GTP and the mixture of non-radioactive amino acids from Calbiochem (San Diego, Calif., U. S. A.), phosphoenolpyruvic acid and pyruvate kinase from Boehringer (Mannheim, G. F. R.), puromycin from Sigma (St. Louis, Mo., U, S. A.), cycloheximide (Actidione) from Carl Roth (Karlsruhe, G. F. R.,) and actinomycin D from Merck, Sharp & Dohme (Rahway, N. Y., U. S. A.). CM-Sephadex A-50 and Sephadex G-200 were products of Pharmacia (Uppsala, Sweden), and DEAE-cellulose Whatman DE-11, of Balston Ltd (Maidstone, Kent, England).

RESULTS

Soluble and mitochondrial tyrosine aminotransferase in frog liver

In frog liver, the subcellular distribution of tyrosine aminotransferase was the same as reported by Miller & Litwack (1971) for the rat. Almost the whole activity was present in the soluble fraction and only 3% in mitochondria (Table 1). The so-

Table 1

Tyrosine aminotransferase activity in rat and frog liver

The activity was determined under standard conditions, with 0.8 - 1.2 mg protein. Mean values from 5 - 10 experiments ± S.D. are given.

Animal	Fraction	Activity (nmoles/min/mg protein)	
		full sample	PLP omitted
Frog	30 000 *g* supernatant	10 ± 1.2	8.6 ± 0.9
	Mitochondria	2 ± 0.3	2 ± 0.3
Rat	30 000 *g* supernatant	11 ± 1.3	

luble aminotransferase was practically saturated with the coenzyme, as omission of PLP decreased its activity only by 15%, whereas omission of PLP had no effect on the mitochondrial enzyme. The two enzymes differed in specificity toward the acceptor of the amino group from tyrosine. The soluble enzyme showed high specificity for 2-oxoglutarate whereas the mitochondrial enzyme exhibited 30% of activity in the presence of pyruvate and 20% with oxaloacetate (Table 2).

Table 2

Specificity of tyrosine aminotransferase from frog liver towards 2-oxoacids

The activity was determined in the presence of the indicated 2-oxoacid at 1 mM concentration, in the standard incubation mixture. Mean values of 3 experiments are given.

Fraction	Activity (nmoles/min/mg protein)		
	2-Oxoglutarate	Oxaloacetate	Pyruvate
30 000 *g* supernatant	10.3	0.56	0.46
Mitochondria	2	0.4	0.6

The activity of tyrosine aminotransferase in frog liver shows season-dependent differences (see Table 4). In winter (October through April) the activity was low and corresponded to about 1/3 of the activity observed in summer (May through September). All the experiments were carried out in summer.

On DE-11 cellulose chromatography, the activity of soluble aminotransferase was resolved into two peaks, a small one eluted with the buffer, and a main one emerging at 0.2 M-NaCl (Fig. 1). The same resolution pattern was obtained on CM-Sephadex column. On polyacrylamide gel at pH 7.6 the main fraction migrated towards anode. The mitochondrial enzyme gave only one peak which was eluted from DE-11 cellulose with buffer alone, and on electrophoresis under the above conditions remained at the start.

The approximate molecular weight of the soluble aminotransferase was 115 000 daltons (Fig. 2), i. e. the same value as for the enzyme from rat liver (Valeriote *et al.*, 1969).

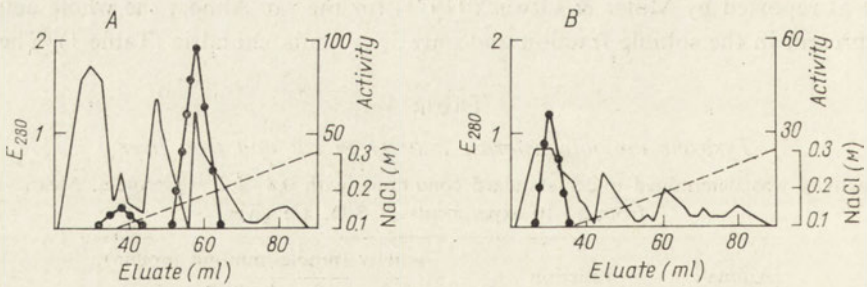

Fig. 1. DE-11 cellulose chromatography of tyrosine aminotransferase from *A*, 30 000 *g* supernatant, and *B*, mitochondrial fraction of frog liver. The amount of protein applied to the column was 80 and 60 mg, respectively. For details see Methods. Fractions of 3 ml were collected. ——, E_{280}; ●, enzyme activity (nmoles pHPP/min/ml).

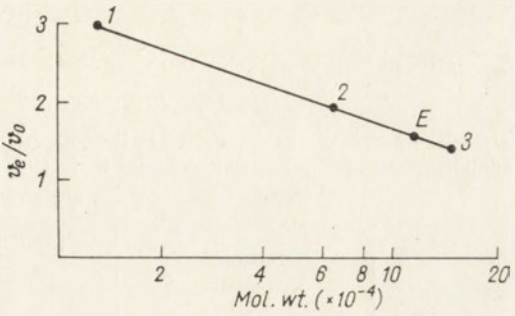

Fig. 2. Molecular weight determination of purified soluble tyrosine aminotransferase from frog liver, by gel filtration on Sephadex G-200. The column (1.4 × 40 cm) was equilibrated with 50 mM-K-phosphate buffer, pH 6.8. The standard proteins, dissolved in the same buffer (5 mg each) were: *1*, cytochrome *c* (mol. wt. 13 000); *2*, bovine serum albumin (mol. wt. 67 000); *3*, rabbit γ-globulin (mol. wt. 150 000). *E*, tyrosine aminotransferase.

Both aminotransferases from frog liver are very similar to the enzymes of rat liver. Miller & Litwack (1971) demonstrated that in the rat the soluble enzyme is an anionic protein, non-saturated with coenzyme, and specific for 2-oxoglutarate, whereas the mitochondrial enzyme, present in much smaller amounts, is a cationic protein saturated with coenzyme, showing low specificity towards the amino group acceptor.

Induction of tyrosine aminotransferase in frog liver

The effect of intraperitoneal injection of insulin, epinephrine, glucagon, cyclic AMP or triiodotyronine, on the activity of tyrosine aminotransferase in liver of frog and rat, is presented in Table 3. In the rat, all the compounds studied, in agreement with the data reported in the literature, enhanced the enzyme activity, whereas in the frog stimulation was observed only on administration of triiodotyronine. Unexpectedly, insulin and cAMP caused a 50% and 70%, respectively, decrease in activity. Glucagon and epinephrine were ineffective. It should be noted that the stimulating effect of triiodotyronine was observed in the frog only in summer; in winter, when the enzyme activity was low, triiodotyronine had no effect (Table 4).

Table 3

Effect of hormones on the activity of tyrosine aminotransferase in the liver of frog and rat in vivo

The compounds studied were injected intraperitoneally in the morning in the amounts indicated in parentheses, and after 4 h the animals were killed. The activity was determined in the 30 000 *g* supernatant in the standard incubation mixture. Mean values ± S.D. are given.

Compound administered (per 1 g body wt.)	Frog		Rat	
	nmoles/min/g liver	% of control	nmoles/min/g liver	% of control
Control	1970 ± 197		4 260 ± 503	
Insulin (2.5 m-units)	900 ± 47	49	7 830 ± 510	185
Epinephrine (0.7 μg)	1920 ± 165	104	10 200 ± 560	240
Glucagon (5 μg)	1920 ± 173	104	7 200 ± 530	168
cAMP (5 μg)	536 ± 32	30	6 170 ± 490	145
Triiodotyronine (3 μg)	6600 ± 720	338	5330 ± 525	125

Table 4

Effect of triiodotyronine in vivo *on the activity of soluble tyrosine aminotransferase in frog liver, depending on the season*

The enzyme activity was determined in the liver 30 000 *g* supernatant from animals killed 4 h after administration of triiodotyronine (3 μg/g body wt.). For details see Methods. Mean values ± S.D. are given.

Season	Control	Triiodotyronine-treated
	Activity (nmoles/min/g liver)	
Summer (May - September)	1960 ± 197	6600 ± 720
Winter (October - April)	600 ± 40	590 ± 63

In summer, within two hours after administration of triiodotyronine, the aminotransferase activity was unchanged, after 4 h the maximum increase was observed, and after 10 h the activity was still higher by a half than the control value (Fig. 3). Actinomycin D and cycloheximide abolished the effect of triiodotyronine.

Johnson *et al.* (1973) reported that the rat liver cytoplasmic tyrosine aminotransferase could be separated on CM-Sephadex chromatography into three fractions eluted at different KCl concentrations. The three forms had the same molecular weight and were immunologically identical. The authors demonstrated that the form eluted at the highest KCl concentration represents the primary product of aminotransferase synthesis and is induced by hormones. This form undergoes reversible interconversion with the other two forms of the enzyme.

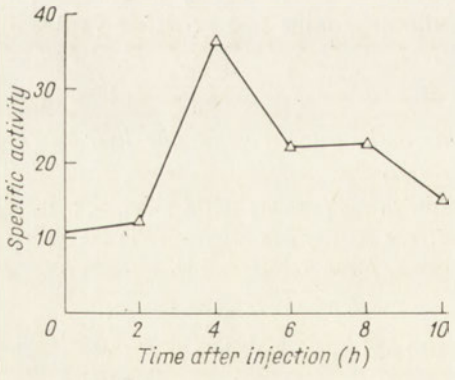

Fig. 3. Time-course of tyrosine aminotransferase activity after triiodotyronine administration (3 μg/g body wt.). The activity was determined in the liver 30 000 *g* supernatant. The results are mean values from 5 experiments, and are expressed as nmoles of pHPP formed/min/mg protein.

In our experiments, the soluble aminotransferase from frog liver, when applied to CM-Sephadex under conditions described by Johnson *et al.* (1973), resolved into two forms (Fig. 4A): a small peak eluted with the buffer, and only a single fraction eluted with the KCl gradient. On induction with triiodotyronine, this form showed a considerable increase (Fig. 4B).

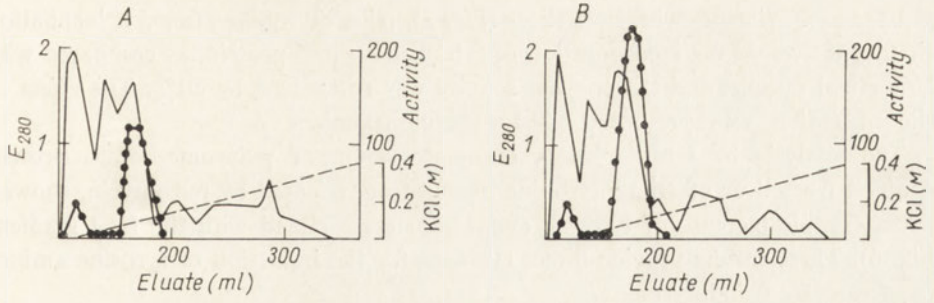

Fig. 4. CM-Sephadex chromatography of tyrosine aminotransferase from the liver of: *A*, untreated frog and *B*, frog treated with triiodotyronine (4 h after injection of 3 μg/g body wt.). To the column, 80 mg of protein was applied. For details see Methods. Fractions of 3 ml were collected. ——, E_{280}; ●, enzyme activity (nmoles pHPP/min/ml).

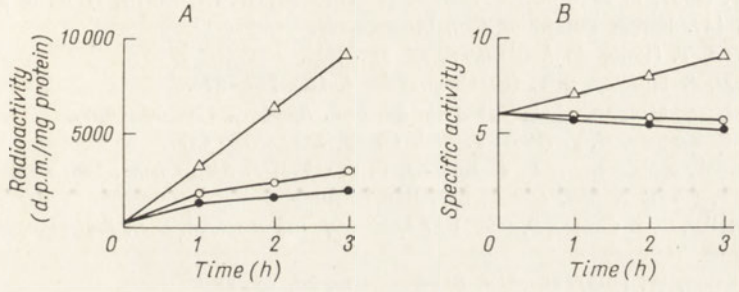

Fig. 5. Effect of triiodotyronine on *A*, incorporation of [^{14}C]leucine into protein, and *B*, tyrosine aminotransferase activity, in frog liver slices. The slices were incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing [^{14}C]leucine (5 μCi/ml) and: △, 0.1 mM-triiodotyronine; ○, triiodotyronine and 1 mM-cycloheximide; ●, control. At 1 h intervals, slices were homogenized and protein, radioactivity and enzyme activity were determined. Specific activity is expressed as nmoles pHPP/min/mg protein. For details see Methods.

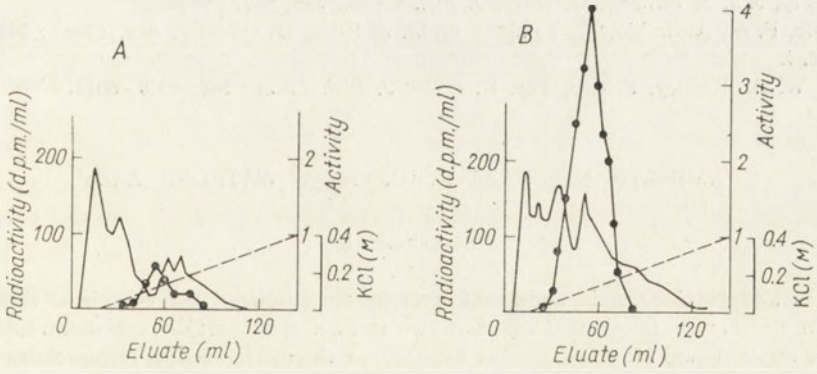

Fig. 6. CM-Sephadex chromatography of tyrosine aminotransferase released from liver polysomes of: *A*, control, and *B*, triiodotyronine-treated frog. Isolated polysomes were labelled for 5 min with [^{14}C]leucine and the polysome-bound protein released by puromycin. The mixture was applied to the CM-Sephadex column (1.2 × 16 cm) and eluted with phosphate buffer, pH 6.8, and a linear KCl gradient. In the eluate, ——, radioactivity and ●, enzyme activity were determined.

In the experiments with liver slices (Fig. 5), triiodotyronine after 3 h incubation stimulated fivefold the incorporation of [^{14}C]leucine into protein, as compared with the control sample, and the enzyme activity was stimulated by 60%. The effect of triiodotyronine was prevented by 1 mM-cycloheximide.

The analysis by CM-Sephadex chromatography of polysome-bound protein obtained from liver of triiodotyronine-treated frog, released by puromycin, showed a considerable increase of tyrosine aminotransferase eluted with the KCl gradient (Fig. 6). These results provide direct evidence for the induction of tyrosine aminotransferase by triiodotyronine.

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AMINOTRANSFERAZA TYROZYNY W WĄTROBIE ŻABY

Streszczenie

1. W wątrobie żaby aminotransferaza tyrozyny zlokalizowana jest głównie w cytoplazmie komórki. Enzym rozpuszczalny jest specyficzny wobec 2-ketoglutaranu; jest on białkiem anionowym o masie cząsteczkowej 115 000 daltonów. Rozdzielono chromatograficznie rozpuszczalną aminotransferazę na dwie formy aktywne o różnym powinowactwie do CM-Sephadex.

2. Trójjodotyronina indukuje aktywność aminotransferazy w wątrobie żaby *in vivo*. Adrenalina i glukagon są bez wpływu, a cAMP i insulina obniżają aktywność enzymu.

3. Trójjodotyronina stymuluje włączanie [^{14}C]leucyny do białek i biosyntezę aminotransferazy na polisomach wątroby żaby.

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ROLE OF Mn^{2+} IN THE REACTION OF POLYNUCLEOTIDE PHOSPHORYLASE WITH 2′-*O*-METHYLATED SUBSTRATES*

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Polymerization of 2′-*O*-methylcytidine-5′-diphosphate (CmDP) with polynucleotide phosphorylase in the presence of Mn^{2+} proceeds with 65% yield after 72 h, and in the presence of Mg^{2+} the yield does not exceed 10%. Phosphorolysis of poly 2′-*O*-methylcytidylic acid and poly 2′-*O*-methyluridylic acid, as well as exchange of the β-phosphate group of CmDP in the presence of Mn^{2+} and Mg^{2+}, proceed with a yield of only a few percent. A possible mechanism of Mn^{2+} action on CmDP polymerization is discussed.

The ability of polynucleotide phosphorylase (EC 2.7.7.8) to polymerize AmDP[1] (Rottman & Heinlein, 1968), CmDP (Janion *et al.*, 1970) and UmDP (Żmudzka & Shugar, 1971) demonstrated that this enzyme, contrary to previously held views, will tolerate modifications of the sugar residue. It was, furthermore, found that replacement of Mg^{2+} by Mn^{2+} appreciably increased the efficiency of polymerization of substrates with C-2′ substituents (Janion *et al.*, 1970; Janik *et al.*, 1972; Khurshid *et al.*, 1972; Tazawa *et al.*, 1972; Torrence *et al.*, 1972; Hobbs *et al.*, 1972a,b, 1973; Kuśmierek & Shugar, 1973; Kuśmierek *et al.*, 1973). This prompted us to examine the role of Mn^{2+} in the polymerization reaction, as well as to examine the susceptibility of 2′-*O*-methyl polynucleotides to phosphorolysis, and the role of Mn^{2+} in this reaction. It is now well known that 2′-*O*-methyl nucleotides are natural constituents of tRNA and rRNA and the *in vivo* function of polynucleotide phosphorylase is believed to involve degradation, and not synthesis, of nucleic acids.

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[1] The following abbreviations are used: AmDP, 2′-*O*-methyladenosine-5′-diphosphate; CmDP, 2′-*O*-methylcytidine-5′-diphosphate; CmMP, 2′-*O*-methylcytidine-5′-monophosphate; UmDP, 2′-*O*-methyluridine-5′-diphosphate; poly(Cm), poly 2′-*O*-methylcytidylic acid; poly(Um), poly 2′-*O*-methyluridylic acid; PNPase, polynucleotide phosphorylase (EC 2.7.7.8).

MATERIAL AND METHODS

Use was made of commercial preparations of CDP and UDP (Koch-Light Lab. Ltd, Colnbrook, Bucks, England, and Calbiochem, Los Angeles, Calif, U. S. A., respectively), and of chemically synthesized 2′-*O*-methylcytidine-5′-diphosphate containing 10% of the isomer methylated in position 3′, a gift from J. Kuśmierek (Kuśmierek *et al.*, 1973). Concentration of nucleoside pyrophosphates was assayed spectrophotometrically. The polymerization of CmDP was calculated from the content of the CmDP in the preparation, assuming that 3′-*O*-methylcytidine-5′-diphosphate was not incorporated in the presence of PNPase (Kuśmierek & Shugar, 1973). Poly(C), poly(U) and poly(Cm) were obtained from the corresponding nucleoside-5′-diphosphates with PNPase from *Micrococcus luteus* under previously reported conditions (Matthaei *et al.*, 1967; Janion *et al.*, 1970); in 0.1 M-phosphate buffer, pH 7.8, their $s_{20,w}$ values were, respectively, 5.2, 3.8 and 8.0. The sedimentation coefficient of poly(Um) prepared by chemical deamination of poly(Cm) was under the same conditions 9.0 (Żmudzka & Shugar, 1970).

Enzymic studies were performed with polynucleotide phosphorylase from *E. coli* (160 phosphorolysis units per ml) kindly offered by M. Grunberg-Manago.

The course of nucleoside-5′-diphosphate polymerization was followed by measuring the concentration of P_i liberated in 5 µl samples (Chen *et al.*, 1956). The amount of phosphorus formed upon substrate degradation in the control sample (containing no enzyme) did not exceed 10% after 72 h; the control value was each time subtracted from the result. For the analysis of the polymerization products ascending chromatography on Whatman no. 1 paper was also used with isopropanol - 1% ammonium sulphate (3:2, v/v) as the solvent system. The R_F values of poly(Cm), CmDP and CmMP were 0, 0.64 and 0.73, respectively.

The course of polynucleotide phosphorolysis and the exchange of the β-phosphate group in CDP and CmDP were determined by monitoring the amount of $^{32}P_i$ incorporated into the nucleoside-diphosphate fraction (Grunberg-Manago *et al.*, 1956), after removal of unreacted P_i according to Berenblum & Chain (1938). Phosphorolysis was performed under conditions applied by Littauer & Kornberg (1957) for phosphorolysis of polyribonucleotides. After the indicated time intervals, samples of 0.01 ml were withdrawn and added to 0.1 ml of 8% trichloroacetic acid (in an ice-bath). After 10 min the precipitate was centrifuged off and the supernatant was added to a solution containing 0.4 ml of water, 0.25 ml of 5% ammonium molybdate and 0.05 ml of 10 M-H_2SO_4. The mixture was extracted twice with 0.8 ml portions of freshly distilled isobutanol, and twice with 0.5 ml of ether, then nitrogen was bubbled through to remove ether. From the solution (0.5 ml) 0.1 ml samples [0.2 ml in the case of poly(U)] were taken and the amount of $^{32}P_i$ was measured in the Mark I scintillation counter.

RESULTS

Polymerization. CmDP polymerization by polynucleotide phosphorylase under conditions previously developed for the synthesis of poly(Cm) (Janion *et al.*, 1970), i. e. in the presence of 10 mM-$MnSO_4$, proceeded very slowly as compared with

CDP (Fig. 1). Equilibrium between polymerization and phosphorolysis was not attained even after 72 h incubation. The results of paper chromatography testified to a gradual increase in the non-migrating spot of the polymer and to the absence of oligonucleotides in the reaction mixture. This is consistent with the high sedimentation coefficients and the high degree of homogeneity of the poly(Cm) preparations obtained.

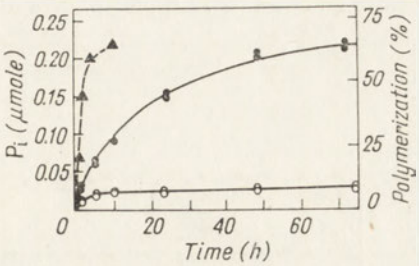

Fig. 1. Effect of Mn^{2+} and Mg^{2+} on CmDP polymerization by polynucleotide phosphorylase. The reaction mixture (0.1 ml) contained: 150 mM-Tris buffer, pH 8.5; 10 mM-$MnSO_4$ (●) or 5 mM-$MgCl_2$ (○); 0.4 mM-EDTA; 1 mM-NaN_3; 7.4 mM-CmDP, and 0.032 phosphorolysis unit of PNPase. The reaction mixture was incubated at 37°C. Polymerization of CDP in the presence of Mn^{2+} (control) (▲).

The yield of CmDP polymerization calculated from the amount of polynucleotide obtained in the reaction on a preparative scale, was dependent on the concentration of Mn^{2+} in the incubation mixture. In the presence of 10, 5, 1 and 0.5 mM-Mn^{2+}, the yield decreased gradually from 65% to 60, 34, and 29%. Under the polymerization conditions employed, considerable amounts of Mn-containing compounds precipitated concomitantly from the alkaline reaction medium. The solubility product of $Mn(OH)_2$ is 4×10^{-14}. Since in the available literature no data on the solubility of manganous phosphate were found, we performed an approximate turbidimetric evaluation, which showed that the concentration of manganous phosphate at pH 8.0 did not exceed 0.06 mM.

CmDP polymerization in the presence of Mg^{2+} attained equilibrium after 10 h, with a yield of only a few percent.

Phosphorolysis. Degradation of poly(Cm) and poly(Um) by polynucleotide phosphorylase was checked under conditions described by Littauer & Kornberg (1957).

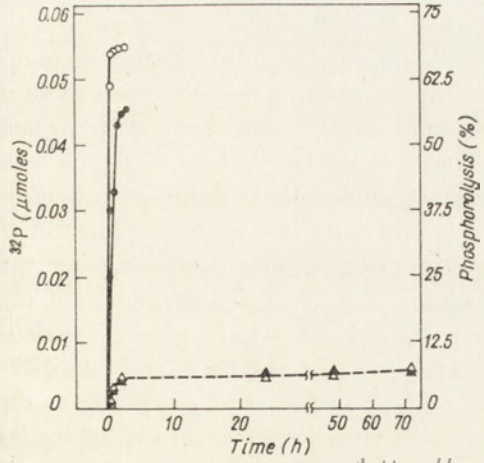

Fig. 2. Phosphorolysis of poly(Cm), poly(Um), poly(C) and poly(U) by polynucleotide phosphorylase. The reaction mixture (0.125 ml) contained: 40 mM-Tris buffer, pH 8.0; 10 mM-phosphate buffer, pH 7.35; 0.5 mM-$MnCl_2$; 0.64 mM-poly(C) (●) or poly(U) (○); or 1.92 mM-poly(Cm) (▲) or poly(Um) (△); ^{32}P-labelled orthophosphate [2×10^6 c.p.m. in sample with poly(C), poly(Cm) and poly(Um), and 0.8×10^6 c.p.m. in sample with poly(U)]; and 0.008 phosphorolysis unit of PNPase. The reaction mixture was incubated at 37°C.

Under these conditions, in the presence of Mg^{2+}, phosphorolysis of poly(C) and poly(U) used as control yielded, in agreement with the results of other authors (Grunberg-Manago, 1959; Littauer & Kornberg, 1957), 81 % and 41 %, respectively, whereas phosphorolysis of poly(Cm) did not exceed 1 %. On the other hand, in the presence of Mn^{2+} (Fig. 2) the yield of phosphorolysis of poly(C), poly(U), poly(Cm) and poly(Um) after 2 h amounted to, respectively, 53, 68, 4 and 4 % and on prolongation of the reaction to 72 h phosphorolysis of poly(Cm) and poly(Um) increased to 7 %.

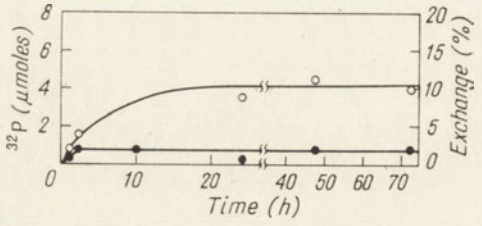

Fig. 3. The exchange of β-phosphate group in CmDP and CDP by polynucleotide phosphorylase. The reaction mixture (0.12 ml) contained: 83.5 mM-Tris buffer, pH 8.0; 4.6 mM-phosphate buffer, pH 7.35; 0.46 mM-$MnCl_2$; 3.16 mM-CDP (○) or CmDP (●); $^{32}P_i$ (1×10^6 c.p.m. and 1.25×10^6 c.p.m. with CmDP and CDP, respectively), and 0.023 phosphorolysis unit of PNPase.

Exchange of β-phosphate group. The exchange reaction catalysed by polynucleotide phosphorylase in the presence of Mn^{2+} showed a yield of about 2 % either on 2 or 72 h incubation (Fig. 3), and 11 - 12 % (after 72 h) with CDP used as control. The corresponding values obtained in the presence of Mg^{2+} (not shown in the Figure) were 47 % for CDP and 1 % for CmDP, which is in agreement with the results of Simuth *et al.* (1971)

DISCUSSION

Our results concerning CmDP polymerization, phosphorolysis of poly(Cm) and poly(Um) and the exchange of β-phosphate group of CmDP demonstrated that the 2′-*O*-methylated derivatives proved to be very inefficient substrates of polynucleotide phosphorylase, as compared with CDP, poly(C) and poly(U). The replacement of Mg^{2+} by Mn^{2+} resulted in a considerable increase of the polymerization yield, up to 65 %, but had practically no effect either on phosphorolysis or exchange of the β-phosphate group.

The role played by manganese in the enzymatic reactions of polynucleotide phosphorylase has not hitherto been elucidated. When analysing this reaction, one must realize that Mn^{2+} differs from Mg^{2+} in the ionic radius, presence of *d* electrons and shape of the external orbitals. It may be assumed therefore that Mn^{2+} interacts with the substrate, especially with its phosphate group, differently from Mg^{2+}, or induces a different conformation of the enzyme. This may result in a specific orientation of the substrate orbitals at the active center of the enzyme, and compensate for the lack of specific interaction between the enzyme and the modified substrate, i. e. the 2′-*O*-methylated compounds. In addition, consideration has to be given to dissimilarities in the properties of salts and bases of these two cations, for instance the differences in their solubilities and the ease of oxidation of divalent manganese to the trivalent form. The low solubility of manganous phosphate seems to be of special

importance, since it exerts an effect on the concentrations of both phosphoric acid and manganese.

Under the experimental conditions employed in the study on phosphorolysis of poly(Cm) and poly(Um) and exchange of the β-phosphate group of CmDP, the phosphate concentration was 25 and 10 times higher, respectively, than that of manganous salt ($MnSO_4$ was added to the reaction mixture in the same concentration as $MgCl_2$). Therefore precipitation of manganous phosphate only slightly changed the total phosphate concentration. In addition, a similar course of poly(C) and poly(U) phosphorolysis, and that of the exchange of the β-phosphate group of CDP in the presence of Mg^{2+} or Mn^{2+}, indicated that the amount of manganese remaining in the solution was sufficient for the cofactor function of Mn^{2+}. Consequently, it is not at all surprising that both these processes involving the 2′-*O*-methylated substrates proceeded similarly, irrespective of the cofactor used. However, in the case of polymerization, at the beginning of the reaction the solution was saturated with Mn^{2+}, whereas the concentration of inorganic phosphate was nearly zero. With progressing polymerization, the phosphoric acid liberated precipitated as manganous phosphate while manganous compounds occurring in the insoluble phase permitted the maintenance of a constant Mn^{2+} concentration in the solution. Thus, the effect of Mn^{2+} on polymerization was probably related to removal of phosphoric acid from the medium, shifting thus the reaction towards polymerization[2]. This explains the high yield (65%) of CmDP polymerization. At the same time, the resistance of 2′-*O*-methylpolynucleotides to nucleases (Rottman & Heinlein, 1968; Janion *et al.*, 1970) as well as the assumption that the polymerization of modified substrates — like the natural ones — proceeds without removal of the growing chain from the enzyme between the successive polymerization steps ("non-synchronous" or "proccessive mechanism", Godefroy-Colburn & Grunberg-Manago, 1972) explain the high molecular weight and homogeneity of the resulting polymers.

Special attention ought to be given to the resistance of poly(Cm) and poly(Um) to phosphorolysis, since under the conditions of the reaction these compounds, like poly(C) and poly(U), exhibit no ordered secondary structure which could hinder this process (Grunberg-Manago, 1959; Żmudzka *et al.*, 1969; Żmudzka & Shugar, 1970). It may be assumed that the presence of 2′-*O*-methylated nucleotides, or of their sequences in rRNA, tRNA, snRNA, hnRNA and mRNA can hinder phosphorolysis of these acids *in vivo* and *in vitro*.

However, it is known that exhaustive *in vitro* phosphorolysis, in the presence of polynucleotide phosphorylase, of tRNA segments containing 2′-*O*-methylated nucleotides, as well as of whole rRNA molecules bring about complete degradation of these preparations (Godefroy-Colburn & Grunberg-Manago, 1972). The source of this discrepancy is not clear. It is possible that 2′-*O*-methylated nucleotides only decreased the rate of phosphorolysis of tRNA and rRNA.

[2] A similar shift towards polymerization of CDP and CmDP with the yields of 40 and 35%, respectively, was observed in the presence of Ca^{2+} due to precipitation of calcium phosphate.

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ROLA Mn^{2+} W REAKCJACH POLINUKLEOTYDOWEJ FOSFORYLAZY Z 2′-*O*-METYLOWANYMI SUBSTRATAMI

Streszczenie

Polimeryzacja 2′-*O*-metylocytydyno-5′-dwufosforanu (CmDP) z polinukleotydową fosforylazą w obecności Mn^{2+} zachodzi z wydajnością 65% po 72 godz., a w obecności Mg^{2+} wydajność nie przekracza 10%. Fosforoliza poli 2′-*O*-metylourydylowego kwasu i poli 2′-*O*-metylocytydylowego kwasu, jak również wymiana β-fosforanowej grupy w obecności Mn^{2+} i Mg^{2+} zachodzi z wydajnościami sięgającymi kilku procent. W pracy dyskutowana jest rola manganu w procesie polimeryzacji CmDP.

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OXIDATION PROCESSES AND UBIQUINONE LOCALIZATION IN THE BRANCHED RESPIRATORY SYSTEM OF *mi-1* MUTANT OF *NEUROSPORA CRASSA*

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1. Stimulation of succinate oxidation in *mi-1* mitochondria by Mg^{2+} and P_i is abolished on uncoupling, which points to the energy-linked activation of succinate oxidation.

2. Mitochondria exhibited respiratory control with succinate and NADH when the cyanide-insensitive oxidation was inhibited by salicylhydroxamic acid (SHAM). SHAM lowered the oxidation rate with NADH and succinate to the same level, though the NADH oxidation rate was 2.5 times as high as with succinate.

3. Despite the high stimulation of succinate oxidation *via* the SHAM-sensitive pathway in the active and controlled state of mitochondria, the redox state of UQ in all metabolic states remains unchanged. On inhibition of the cyanide-insensitive pathway, UQ reduction is greatly increased only in the controlled and active state. With NADH as a substrate, UQ does not respond to the metabolic states of mitochondria.

4. The redox changes of cytochrome *c* parallel those of UQ.

5. Branching of the respiratory chain in *mi-1* mitochondria is discussed.

In recent years a number of reports have shown that all types of cytochromes present in the wild type of *Neurospora crassa* and in mammalian mitochondria occur also in *mi-1* mutant (*poky*). The difference is only of a quantitative nature and concerns cytochromes *b* and $a+a_3$, the content of which in young *mi-1* is either none or extremely low, and increases gradually with the age of the culture (Haskins *et al.*, 1953; Edwards & Woodward, 1969; Lambowitz *et al.*, 1972a,b; Jagow *et al.*, 1973). Another striking difference is the molar ratio of b_T:b_K, the two components of cytochrome *b*, which in wild type is 1:1 and in *mi-1* mutant 1:6 (Jagow *et al.*, 1973). The two other components: cytochrome *c* and UQ, accumulate in a much higher

* This work has been done during a short-term stay at the Institut für Physiologische Chemie und Physikalische Biochemie der Universität München.

amount in the mutant than in the wild type (Haskins *et al.*, 1953; Drabikowska & Kruszewska, 1972).

The respiratory system of *mi-1* mutant is only partly cyanide- and antimycin A-sensitive. The major part of electron flow is channeled *via* a cyanide-insensitive pathway which is inhibited by salicylhydroxamic acid (SHAM)[1], a specific inhibitor of a similar cyanide-insensitive respiration in plant mitochondria (Lambowitz & Slayman, 1971; Schonbaum *et al.*, 1971).

Lambowitz & Slayman (1971) have suggested that the respiratory system of *mi-1* is branched, with the branching point located before cytochromes. Recently, Jagow *et al.* (1973) postulated that UQ could serve as a component transferring electrons from the flavin region both to the cytochrome system and to a cyanide-resistant pathway.

Our studies on oxidation of NADH and succinate in *mi-1* included uncoupled, active and controlled states of mitochondria. The redox changes in UQ and cytochrome *c* were concomitantly recorded. The results obtained seem to suggest branching of the respiratory chain at the level of flavins.

MATERIALS AND METHODS

Growth of cells. Strain *mi-1 nic-2a* (FGSC 1577) was kindly sent by Fungal Genetics Stock Centre, Humboldt State College, Arcata (Calif., U. S. A.). The mycelium was grown from conidia (2.8×10^8) in the aerated Vogel minimal medium supplemented with 2% sucrose and 10 mg nicotinamide per 1 litre, at 34°C. After 24 h the mycelium was harvested and washed with water, whereupon the excess of water was squeezed out.

Preparation of mitochondria. Hyphae were suspended in 5 vol. of the medium consisting of 0.44 M-sucrose, 2 mM-EGTA, 10 mM-Tris-acetate and 0.2% of albumin, pH 7.3 (Weiss *et al.*, 1970) and disrupted in a grind mill at 0 - 4°C. The homogenate was centrifuged at 1500 *g* for 3 min. The resulting supernatant was filtered through a double layer of cheese-cloth and centrifuged once more at 1500 *g* for 10 min. The pellet was discarded, the mitochondria were sedimented at 10 000 *g* for 30 min and suspended in the initial medium to contain 30 - 40 mg protein per 1 ml.

Respiration measurements. Respiration was measured polarographically using Clark-type oxygen electrode. The reaction medium was of the same composition as that used for the isolation of mitochondria. The medium was saturated with air before each measurement. A concentration of 240 nmoles of oxygen per 1 ml of the medium was taken for calculation. Experiments were carried out at 22°C.

Determination of redox changes of cytochrome c. Redox changes of cytochrome *c* were assayed in the above-mentioned medium using a dual-wavelength spectrophotometer. $\Delta E_{550-540\ nm}$ was recorded. Protein concentration was chosen so as to avoid anaerobiosis during the experiments.

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* Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SHAM, salicylhydroxamic acid; UQ, ubiquinone.

Ubiquinone determination. Ubiquinone content in mitochondria was determined by the extraction method of Kröger & Klingenberg (1966). The extracted ubiquinone was determined spectrophotometrically (in a double-beam spectrophotometer) from the decrease in absorbance at 280 - 288 nm following the addition of KBH_4. The absorbance coefficient (oxidized *minus* reduced) of 8800 moles$^{-1} \times$ cm^{-1} was used for calculation.

Determination of protein. Protein was estimated by the biuret method as described by Szarkowska & Klingenberg (1963). Protein content was calculated from the decrease in absorbance after addition of cyanide, according to the formula: mg protein $= A_{546\ nm} \times 17.5$, for the volume of 5 ml and light-path of 1 cm.

RESULTS

Oxidation of succinate. Mitochondria of *mi-1* mutant of *N. crassa* suspended in the medium used for isolation, oxidize succinate at a relatively low rate (Table 1). Presence of both Mg^{2+} and P_i is required for the maximum activity (Drabikowska *et al.*, 1974). Upon addition of ADP, no respiratory control can be noticed. Uncoupling brings about a great decrease in the overall oxidation rate of succinate.

Table 1

Effect of Mg^{2+} *and* P_i, *FCCP, ADP and SHAM on the succinate and NADH oxidation in* mi-1 *mitochondria*

Oxygen consumption was measured polarographically under conditions described in Materials and Methods. Where shown, 2 mM-SHAM, 1 μM-FCCP, 5 mM-$MgSO_4$, 5 mM-phosphate, 0.8 mM-ADP and 10 mM-succinate or 1 mM-NADH were used. Concentration of mitochondrial protein was 0.6 mg/ml and 0.3 mg/ml in the experiments with succinate and NADH, respectively. SHAM was added 2 min before addition of the substrate. The reaction was run at 22°C.

Additions	Oxygen consumption (nmoles/mg protein/min)	
	NADH	succinate
None	405	95
FCCP	—	72
SHAM	50	50
$Mg^{2+}+P_i$	410	162
$Mg^{2+}+P_i+ADP$	410	162
$Mg^{2+}+P_i+FCCP$	400	85
$Mg^{2+}+P_i+FCCP+SHAM$	75	70
$Mg^{2+}+P_i+SHAM$	50	50
$Mg^{2+}+P_i+SHAM+ADP$	77	75
$Mg^{2+}+P_i+KCN$	355	107

When the cyanide-resistant oxidation is inhibited by SHAM, the rate of succinate oxidation *via* the cytochrome pathway is independent of the presence or absence of Mg^{2+} and P_i. These results indicate that both these ions stimulate succinate oxidation *via* the SHAM-sensitive pathway. Furthermore, in the presence of SHAM mi-

tochondria show respiratory control of 1.6 on addition of ADP and a slight stimulation of oxidation in the presence of the uncoupler FCCP (Fig. 1, Table 1). Oxidation *via* the cytochrome pathway is stimulated upon addition of ADP, whereas the total oxidation rate remains unchanged. Since the total oxidation activity is the sum of both pathways (Table 1), oxidation *via* the SHAM-sensitive pathway must be decreased. From these results it is also evident that the cytochrome respiratory system in *mi-1* mitochondria responds to uncouplers and ADP similarly as that occurring in mitochondria of mammals and the wild type *N. crassa*.

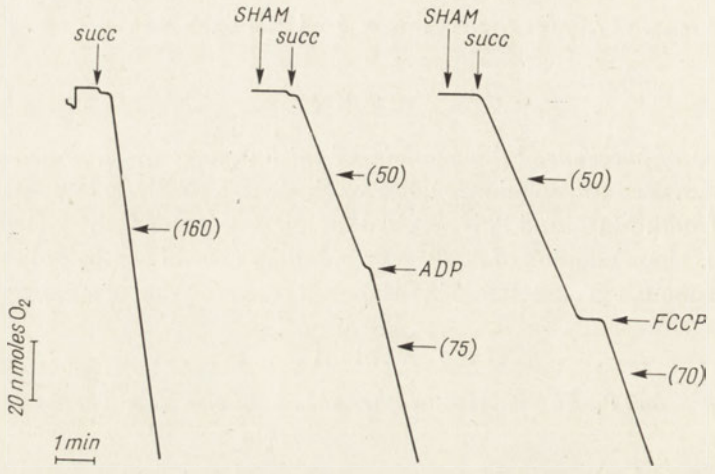

Fig. 1. Respiratory pattern of succinate oxidation in *mi-1* mitochondria. Conditions of reaction and composition of medium were as described in the legend to Table 1. The incubation medium was supplemented with 5 mM-$MgSO_4$ and 5 mM-phosphate. Concentration of mitochondrial protein was 0.6 mg/ml.

Oxidation of exogenous NADH. Exogenous NADH is very actively oxidized by mitochondria of young *mi-1* mutant of *N. crassa*, in contrast to animal mitochondria which are impermeable to this substrate. In uncoupled mitochondria the oxidation of NADH is usually 3 - 6 times higher than that of succinate. SHAM decreases by 84 - 90% oxidation of NADH in young *mi-1* (Table 1). When the cyanide-insensitive respiration is blocked by SHAM, the mitochondria show respiratory control on addition of ADP similarly as in the case of succinate.

In contrast to the oxidation of succinate, NADH oxidation remains unaffected by uncoupling. Moreover, the NADH-oxidizing system in *mi-1* mitochondria is only insignificantly, if at all, stimulated by Mg^{2+} and P_i (Table 1).

Redox reactions of UQ. The reduction of mitochondrial UQ by succinate and NADH is given in Table 2. The results refer to the same mitochondrial preparation used in the experiments presented in Table 1. In the absence of inhibitors, the degree of UQ reduction depends upon the oxidizable substrate present. Thus, in the presence of NADH, which is actively oxidized, the UQ reduction is very high (60 - 70%), whereas it is much lower with the slowly oxidized succinate (20 - 35%).

Table 2

The relative redox state of ubiquinone in aerobiosis and in the presence of inhibitors

The *mi-1* mitochondria were suspended in the isolation medium supplemented with 5 mM-$MgSO_4$ and 5 mM-phosphate. The active state of oxidation was initiated by addition of 0.8 mM-ADP. For uncoupling 1 μM-FCCP was used. 1 mM-KCN and 2 mM-SHAM were added 2 min before substrate addition. Mitochondria (0.7 mg protein/ml) were incubated with 1 mM-NADH for 5 sec at room temp. NADH was oxidized at a rate of 405 nmoles O_2/mg protein/min. In the presence of SHAM the rate of oxidation amounted to 50 nmoles O_2/mg protein/min. UQ concentration was 3.1 nmoles/mg protein. Reactions with succinate were run for 15 sec under the same conditions as in the case of NADH except that 10 mM-succinate was used as substrate. The concentration of protein was 1.1 mg/ml. Succinate was oxidized at a rate of 162 nmoles O_2/mg protein/min. In the presence of FCCP the rate of succinate oxidation was 85 nmoles O_2/mg protein/min. SHAM decreased oxidation to 50 nmoles O_2/mg protein/min. The reduction state of ubiquinone under aerobic conditions was taken as 100.

Additions	Metabolic state of mitochondria		
	active	controlled	uncoupled
NADH	66	68	70
SHAM+NADH	88	86	84
KCN+NADH	63	67	74
KCN+SHAM+NADH	88	86	90
Succinate	33	33	35
SHAM+succinate	71	71	44
KCN+succinate	50	48	54
KCN+SHAM+succinate	85	83	86

There is no redox change of UQ on transition from active to controlled or uncoupled states, both with NADH and with succinate, though the oxidation of succinate is greatly decreased by uncouplers. This lack of redox changes of UQ with succinate may be explained only by assuming that the branching point is before UQ. Although the total rate of succinate oxidation is lowered by about 50% upon uncoupling (as compared to the rate in the controlled state of respiration), the rate of oxidation *via* the cytochrome pathway remains constant (Table 1). Thus, the same number of reducing equivalents transferred to UQ must produce the same degree of reduction. Although in the active state the oxidation rate *via* the cytochrome pathway increases, ADP stimulates probably not only the succinate dehydrogenase which donates reducing equivalents to UQ but also some other components which take part in its oxidation; thus, the redox state of UQ may remain unchanged.

In the presence of cyanide, UQ reduction with NADH as substrate is not further enhanced. There is, however, a visible redox change when succinate is used as substrate. In the presence of cyanide only the cytochrome oxidase is inhibited. An interaction between components of the cytochrome respiratory chain and an alternate oxidase still exists. The lack of further reduction of UQ with NADH under these conditions may be explained by a shift of the redox equilibrium between UQ (as acceptor), a dehydrogenase (as a donor of the reducing equivalents to UQ and to the cyanide-insensitive pathway), and an alternate oxidase. This new equilibrium

does not affect the redox state of UQ, since the slow oxidation of highly reduced UQ by the inefficient cytochrome system is of no great importance. The high rate of UQ reduction with NADH and the great excess of UQ, as compared with cytochrome *b*, must also be taken into consideration. Oxidation by the inefficient cytochrome system affects the redox state of UQ when slowly oxidized succinate is used as substrate; this manifests itself by an increase in UQ reduction upon addition of cyanide.

In the presence of SHAM the maximum level of UQ reduction is achieved with NADH as substrate. This inhibition brings about an additional reduction which is only by 20% greater than that in the non-inhibited state and does not reflect the very high oxidation rate of the SHAM-inhibited cyanide-insensitive oxidase (350 nmoles of O_2/mg protein/min are consumed by the SHAM-sensitive oxidase, and only 55 nmoles by the cyanide-sensitive oxidase, cf. Table 1).

In the presence of SHAM, succinate causes a small increase in UQ reduction in the uncoupled state and a very high increase in the active and controlled states. These results indicate that the degree of UQ reduction depends upon the dehydrogenase activity and not upon the activity of the alternate SHAM-sensitive oxidase. Uncoupling of oxidation from phosphorylation decreases the succinate dehydrogenase activity but not the alternate oxidase activity, since under the same conditions NADH is efficiently oxidized by this pathway (Table 1). The difference between the activity of succinate dehydrogenase and of the cytochrome system determines the redox state of UQ when the dehydrogenase has no possibility to supply the reducing equivalents to the alternate oxidase pathway.

Redox reactions of cytochrome c. The UQ redox changes in the branched respiratory chain of *mi-1* mutant were compared with those of cytochrome *c* as the only

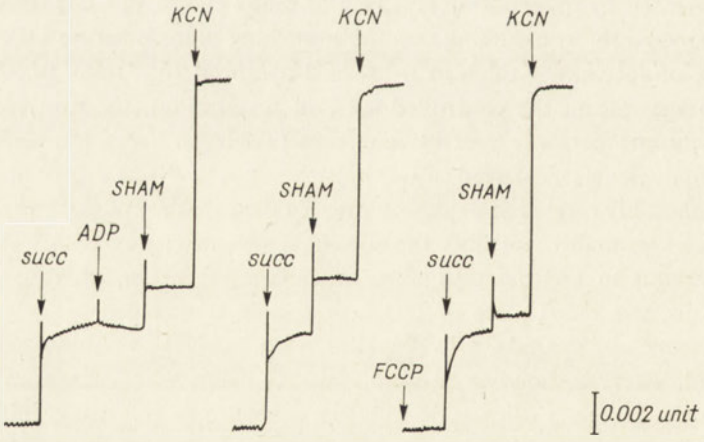

Fig. 2. Redox states of cytochrome *c* in *mi-1* mitochondria in the presence of succinate. The reaction was run in the isolation medium supplemented with Mg^{2+} (5 mM) and P_i (5 mM). Concentration of SHAM and other substances, as described in the legend to Table 1. Redox state of cytochrome *c* was followed spectrophotometrically as described in Materials and Methods. Concentration of mitochondrial protein was 0.46 mg/ml.

component of these mitochondria whose redox changes could be followed spectrophotometrically.

The redox state of mitochondrial cytochrome *c* as well as its responses to respiratory inhibitors in active, controlled and uncoupled states with succinate as substrate, are presented in Fig. 2. The reduction of cytochrome *c* upon addition of succinate amounts to about 26% of the total cytochrome *c* reducible by $Na_2S_2O_4$, irrespective whether mitochondria are uncoupled or coupled. In the presence of SHAM further pronounced reduction occurs only in controlled and active states, similarly as in the case of UQ. Addition of cyanide reduces the whole pool of cytochrome *c* which is involved in electron transport processes.

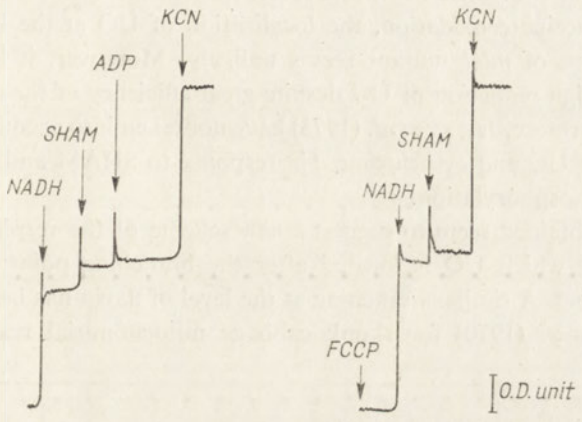

Fig. 3. Redox states of cytochrome *c* in *mi-1* mitochondria in the presence of NADH. Conditions as described in the legend to Fig. 2.

Figure 3 shows the pattern of cytochrome *c* redox changes upon addition of NADH. Unlike succinate, NADH results in the highest reduction of cytochrome *c* in the uncoupled state. Addition of SHAM brings about a further slight reduction. On transition from the controlled to active state, a slight increase only in the reduction of cytochrome *c* is observed. The redox state of cytochrome *c* and its redox changes in the presence of SHAM resemble to some extent the redox changes of ubiquinone under similar conditions. These changes differ, however, in the presence of cyanide.

DISCUSSION

The contribution of cyanide-sensitive oxidation to the overall respiration of *mi-1* mitochondria was found to be of the same order for both NADH and succinate as substrates, though their oxidation rates are different. The most striking property of succinate oxidase in *mi-1* mitochondria is its activation by Mg^{2+} and P_i, and the decrease of its activity on uncoupling. The nature of this activation is not clear; it seems to be connected with some energy-dependent processes, as FCCP abolishes the stimulatory effect of these ions.

The flux of reducing equivalents *via* the cytochrome chain proceeds at the maximal rate, while the contribution of the alternate oxidase varies.

It seems that in young cultures the cyanide-resistant oxidation is of significance for the reoxidation of NADH formed by the oxidative steps of glycolytic processes.

Jagow *et al.* (1973) postulated that the oxidation pathway branches at the level of UQ. However, since those authors used only uncoupled mitochondria, they overlooked the lack of correlation between the UQ redox changes and the rate of succinate oxidation in different metabolic states of mitochondria. If one assumes that UQ is located at the branching point of the respiratory system of *mi-1*, then in controlled and active states the higher is the dehydrogenase activity, the higher should be the reduction of UQ. Since the initial redox state of UQ (see Table 2) is unrelated to the rate of succinate oxidation, the localization of UQ at the branching of the respiratory system of *mi-1* mutant seems unlikely. Moreover, it is not clear why NADH causes high reduction of UQ despite great efficiency of the alternate oxidase (Table 1). Furthermore, Jagow *et al.* (1973) have not taken into account the analogous redox changes in UQ and cytochrome *c* in response to SHAM and to the oxidation controlled by phosphorylation.

The results obtained seem to suggest a new scheme of the respiratory system of *mi-1* (Fig. 4), in which UQ is located after the branching point followed by the cytochrome system. A similar branching at the level of flavin has been postulated by Erecińska & Storey (1970) for skunk cabbage mitochondrial respiratory system.

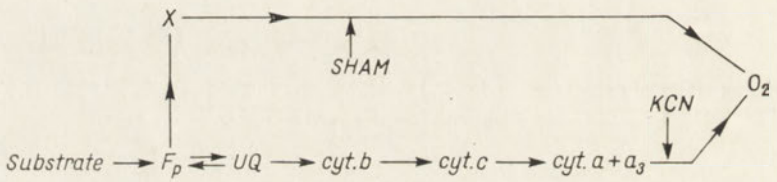

Fig. 4. Proposed scheme of the respiratory system in *mi-1* mutant of *N. crassa*.

In *mi-1* mitochondria the redox state of UQ seems to reflect the donor and acceptor activity of the cytochrome system in terms of Kröger & Klingenberg (1973). However, this picture is complicated by the fact that a certain part of reducing equivalents proceeds *via* the cyanide-insensitive pathway. Thus, one may suspect the existence of an oxidation-reduction equilibrium between the components involved in the electron transport *via* the cyanide-sensitive and cyanide-insensitive pathways. The total amount of reducing equivalents directed to the SHAM-sensitive and cyanide-sensitive pathways may depend upon the dehydrogenase activity, the capacity of both these pathways, and the redox state of UQ, which may have a regulatory function.

The remarkable reduction of UQ in *mi-1* mitochondria linked to the control of oxidative phosphorylation resembles that observed in mammalian mitochondria (Kröger & Klingenberg, 1966).

NADH dehydrogenase saturates with electrons both oxidation pathways to a much greater extent than does succinate dehydrogenase. The UQ redox changes

with NADH, corresponding to low inhibition of oxidation by cyanide, are hardly detectable. An 85% inhibition of oxidation caused by SHAM brings about a further shift of the oxidation-reduction equilibrium of UQ entirely towards the reduced form (Table 2). Reducing equivalents are, under these conditions, supplied by NADH dehydrogenase to the cytochrome system only *via* UQ, since the alternate pathway of electron transport has been cut off.

The localization of UQ in the cytochrome chain is also supported by the comparison of the redox states of cytochrome *c* and UQ. Reduction of cytochrome *c* by either NADH or succinate is lower than the reduction of UQ because cytochrome *b* limits the electron flow from the dehydrogenases, whereas there is no such limitation for UQ. On the other hand, reduced cytochrome *c* is directly oxidized by cytochrome oxidase without participation of any other component(s) of the cytochrome respiratory chain, whereas UQ is oxidized by cytochrome oxidase *via* cytochrome *b* which occurs in a minute amount in young *mi-1* and therefore limits the oxidation of UQ. Thus, taking into account localization of cytochrome *c* and UQ in the same respiratory chain, the redox changes of these two components can be expected to be similar, but not identical.

The most pronounced difference in the redox state of UQ and cytochrome *c* occurs in the presence of KCN. However, this is a result of the presence of a "natural inhibitor", cytochrome *b*, located between these two components.

Bahr & Bonner (1973) suggested for plant mitochondria another mechanism, on the assumption that the branching point consists of at least two separate components, A and B. If these components represent a dehydrogenase and an alternate oxidase, respectively, then the scheme of Bahr & Bonner could be regarded as consistent with the model suggested in this paper.

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PROCESY UTLENIANIA I LOKALIZACJA UBICHINONU W ROZGAŁĘZIONYM ŁAŃCUCHU ODDECHOWYM U MUTANTA *mi-1 NEUROSPORA CRASSA*

Streszczenie

1. Stymulacja utleniania bursztynianu przez Mg^{2+} i P_i w mitochondriach mutanta *mi-1* jest znoszona przez FCCP, co wskazuje na aktywację utleniania bursztynianu zależną od energii.
2. Mitochondria wykazują kontrolę oddechową z bursztynianem i NADH wtedy, kiedy niewrażliwe na cyjanki utlenianie jest zahamowane przez SHAM. W obecności SHAM utlenianie bursztynianu i NADH zachodzi z tą samą szybkością, chociaż bez inhibitora szybkość utleniania NADH jest 2,5-krotnie wyższa od szybkości utleniania bursztynianu.
3. Mimo znacznej stymulacji utleniania niewrażliwego na cyjanki w stanie kontrolowanym i aktywnym, stopień redukcji UQ z bursztynianem jako substratem pozostaje niezmieniony w stosunku do stanu rozkojarzonego. Zahamowanie niewrażliwej na cyjanki oksydazy przez SHAM powoduje znaczny wzrost stopnia redukcji UQ tylko w stanie kontrolowanym i aktywnym. Z NADH jako substratem stopień redukcji UQ nie zależy od stanu metabolicznego mitochondriów.
4. Zmianom oksydoredukcyjnym UQ towarzyszą podobne zmiany oksydoredukcyjne cytochromu *c*.
5. Zaproponowano nowy schemat łańcucha oddechowego u mutanta *mi-1*.

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RADIATION-INDUCED ALDEHYDES IN COLLAGEN

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Aldehydes present in native and irradiated (30 krad) collagen were separated from its enzymic degradation products using molecular-sieve chromatography on Bio-Gel P-2 column.

From the absorption spectra of *N*-methylbenzothiazolone hydrazone derivatives it was concluded that two out of five of the aldehydes separated from the irradiated collagen, were identical with those present in control collagen. The possible origin of three other aldehydes is discussed.

Radiolysis of deoxygenated solution of collagen results in a significant increase in its aldehyde content (Jeleńska & Dancewicz, 1969). However, there is no concomitant decrease in content of ε-amino groups (Jeleńska & Dancewicz, 1972), which in native collagen serve as a source of aldehydes formed in the course of its maturation (Tanzer, 1973).

Since the aldehydes present in native collagen are potential precursors of intra- and intermolecular cross-links (Bailey *et al.*, 1974), the identity, origin, location and possible involvement of the radiation-induced aldehydes in collagen aggregation processes are of interest for radiobiology. The results presented in this paper bring some new information on this subject.

MATERIALS AND METHODS

Preparation of collagen and irradiation procedure. Collagen soluble in neutral salts was prepared from skin of young rats and irradiated in 1.5% solution under nitrogen with X-rays as described by Jeleńska & Dancewicz (1972).

Enzymic degradation of collagen. Collagen fibrils reconstituted from collagen solution by thermal polymerization were separated by centrifugation and subjected to the action of bacterial collagenase according to Bensusan (1969). The lysates were incubated with papain as described by Hill & Schmidt (1962). Next, both proteolytic enzymes were inactivated and the collagen lysate was subjected to the action of pep-

tidase preparation obtained from microsomes of pig kidney according to Wachsmuth *et al.* (1966). The lysates obtained were dialysed against water, lyophilized and separated on Bio-Gel P-2 column.

Molecular-sieve chromatography on Bio-Gel P-2. Degradation products of collagen were separated on 1.2 × 140 cm column of Bio-Gel P-2 according to Bensusan (1969); 1 ml fractions of effluents were collected and analysed.

Analytical methods. Free amino groups were determined using the ninhydrin method of Moore & Stein (1948), sugars by the anthrone method (Dische, 1962), and aldehydes colorimetrically using MBTH[1] (Paz *et al.*, 1965). Protein was measured by the Kjeldahl method using a factor of 5.36 for calculation of the collagen content. U. v. spectra of MBTH derivatives of aldehydes were measured on Unicam SP-800 spectrophotometer (Paz *et al.*, 1965) at pH 1 and 4.

Chemicals. Bacterial collagenase, MBTH and ninhydrin were purchased from Koch-Light Lab. Ltd (Colnbrook, Bucks., England). Papain was a 2× crystallized product of Mann Research Lab. (New York, U. S. A.). Anthrone was obtained from Polskie Odczynniki Chemiczne (Gliwice, Poland).

RESULTS AND DISCUSSION

Figure 1 shows the elution profiles of enzymic degradation products of collagen, non-irradiated (Fig. 1A) and irradiated with 30 krad of X-rays (Fig. 1B). There were two main peaks of ninhydrin-positive products in both preparations of collagen. The products eluted first from the Bio-Gel P-2 column, regarded by Bensusan (1969) as small peptides, occurred in greater proportion in the irradiated collagen. This implies the occurrence of heavier degradation products in irradiated, as com-

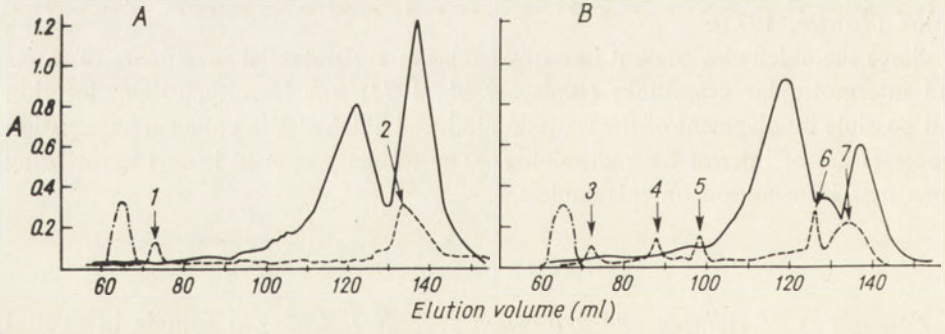

Fig. 1. Elution profiles of enzymic degradation products of collagen, separated on Bio-Gel P-2 column. ——, Ninhydrin-positive products (A_{570}); - - -, products reacting with *N*-methylbenzothiazolone (A_{670}); —·—·—, products reacting with anthrone (A_{620}). Degradation products equivalent to 15 mg of collagen were applied to a 1.9 × 140 cm column and eluted with water. *A*, Native collagen; *B*, collagen irradiated with 30 krad of X-rays.

[1] Abbreviations: MBTH, *N*-methylbenzothiazolone hydrazone; allysine, α-aminoadypic-δ-semialdehyde.

pared with non-irradiated, collagen. The difference could result from the diminished accessibility of the enzymes to collagen due to the radiation-induced changes in molecular conformation and/or the increased number of stable cross-links, which were not susceptible to the enzymic action.

Elution profiles of the products reacting with anthrone were the same in Fig. 1A and 1B. This indicates that no essential change occurred in the reactivity (and inferred integrity) of the sugar moiety in irradiated collagen.

Elution profiles of MBTH derivatives of aldehydes show two peaks (*1* and *2*) in non-irradiated collagen, and five peaks (*3* - *7*) in the irradiated collagen. Peaks *1* and *3*, and *2* and *7* were eluted, respectively, at the same position. Hence, it was assumed that peaks *3* and *7* in the irradiated collagen contain aldehydes which are already present in native collagen, while peaks *4*, *5* and *6* represent aldehydes induced by irradiation.

U. v. spectra of MBTH derivatives of aldehydes from the separated peak fractions (Fig. 2) allowed to identify allysine and α,β-unsaturated aldehyde product of aldol condensation of two allysines. Allysine bound with amino acid or short peptides has the absorption maximum at 308 nm at pH 4. Upon changing pH to 1.0 the absorption maximum is shifted to 298 nm (Bornstein *et al.*, 1966; Bornstein & Piez, 1966; Kang *et al.*, 1969). The same behaviour was observed in the case of aldehydes present in the peak fractions *2*, *6* and *7*. We assumed then that the aldehyde in the peak fractions *2* and *7* showing the same eluting position, was allysine.

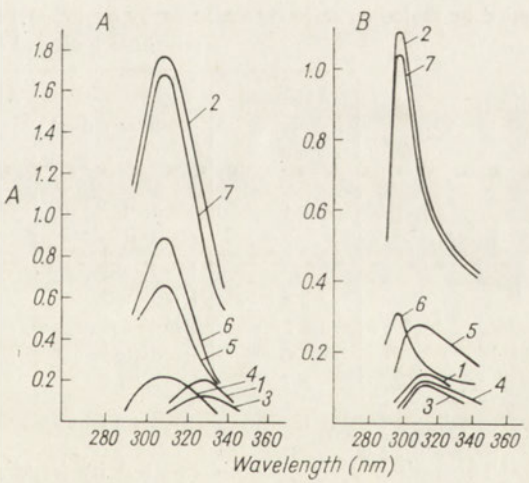

Fig. 2. Absorption spectra of *N*-methylbenzothiazolone hydrazone derivatives of aldehydes, separated from the enzymic degradation products of control collagen (*1* and *2*), and of collagen irradiated with 30 krad of X-rays (*3*, *4*, *5*, *6* and *7*). The numbers refer to the fractions indicated in Fig. 1. Measurements at: *A*, pH 4; *B*, pH 1.

The identity of aldehyde from the peak fraction *6* is uncertain. Optical properties testify to allysine but the eluting position was different. One possibility is that it might be allysine bound to an atypical peptide fragment of the collagen changed by radiation; another, that this is hydroxyallysine.

α,β-Unsaturated aldehyde resulting from the aldol condensation of two allysines has been isolated as a peptide fragment from collagen obtained from different tissues and different species (Bornstein *et al.*, 1966; Butzow & Eichhorn, 1968;

Kang *et al.*, 1969). This aldehyde reacts with MBTH to form a product having the absorption maximum at 330 nm at pH 4, and 312 nm at pH 1. Such optical behaviour was shown by the aldehydes from the peak fractions *1* and *3*. One may conclude, therefore, that irradiated collagen contains the same α,β-unsaturated aldehyde as the native collagen.

Absorption spectra of MBTH derivatives of aldehydes from the peak fractions *4* and *5* differ from that of allysine or α,β-unsaturated aldehyde (Table 1). Upon changing the pH from 4 to 1 there is a red instead of a blue shift of the maximum absorption. The nature and origin of these aldehydes remains unknown in spite of several further attempts undertaken for their identification. Conversion of ε-amino groups to aldehydes due to irradiation seems unlikely, as we have shown that in the irradiated collagen the number of ε-amino groups changes insignificantly (Jeleńska & Dancewicz, 1972). Similarly, $-CH_2OH$ group of a sugar moiety in collagen cannot be regarded as a source of the radiation-induced aldehyde because none of the aldehyde peaks emerged at the position identical with that of the sugar-containing effluent.

Table 1

Absorption maxima of N-methylbenzothiazolone hydrazone derivatives of aldehydes separated from the enzymically degraded collagen

Collagen, control or irradiated with X-rays (30 krad) was degraded step-wise by treatment with bacterial collagenase, papain, and a mixture of four peptidases. Degradation products were separated by molecular-sieve chromatography on Bio-Gel P-2 column. Numbers of fractions analysed are those indicated in Fig. 1.

Aldehyde fraction no.	Collagen used	Absorption maxima (nm) at	
		pH 4	pH 1
1	Control	330	313
2	Control	308	298
3	Irradiated	330	313
4	Irradiated	308	313
5	Irradiated	308	310
6	Irradiated	308	298
7	Irradiated	308	298

The reductive conditions created by irradiation of deoxygenated solution (production of e^-_{aq} and $H^\cdot$ radical) could favour reduction of -COOH to -CHO. To check this possibility, -COOH groups of collagen were esterified with methyl alcohol prior to collagen irradiation. However, the yield of the methylation product amounted only to about 85% which means that some -COOH groups were still free to undergo reduction during irradiation of collagen. Moreover, methylation did not change the amount of the aldehydes induced in collagen by irradiation.

Another approach to identification of the radiation-induced aldehydes in collagen consists in separation of the degradation products containing these compounds,

followed by their structural analysis. Preliminary steps along this line were made by separation, with an amino acid analyser, of the collagen components which are selectively reduced with borohydride. The results obtained (to be published) confirmed the induction of new borohydride-reducible components in collagen due to irradiation. Since aldehydes are most probably the main components which are reducible under the conditions used, it seems that the new reducible components are aldehyde species. Mass-spectrometer analysis of these reducible components would probably help to identify their structure and elucidate their origin.

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ALDEHYDY INDUKOWANE W KOLAGENIE POD WPŁYWEM NAPROMIENIANIA

Streszczenie

Aldehydy występujące w kolagenie natywnym i napromieniowanym promieniami Roentgena (30 kradów) wyizolowano z produktów enzymatycznej degradacji kolagenu, stosując rozdział chromatograficzny na kolumnie z Bio-Gel P-2.

Widma absorpcyjne pochodnych *N*-metylobenzotiazolono-hydrazonowych wykazały, że z pięciu aldehydów wykrytych w kolagenie napromieniowanym, dwa występują w kolagenie kontrolnym. Przedyskutowano możliwe pochodzenie pozostałych trzech aldehydów.

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ALKYLATED CYTOSINE NUCLEOSIDES: SUBSTRATE AND INHIBITOR PROPERTIES IN ENZYMATIC DEAMINATION*

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Cytosine nucleoside deaminase (EC 3.5.4.5) from *Salmonella typhimurium* LT2 catalyses the deamination of ribo-, deoxyribo- and arabinosyl nucleosides of cytosine alkylated at the C-5, but not at the N_3 or exocyclic N^4, of the pyrimidine ring. The enzyme was inert towards analogues etherified at the 3′-OH and 5′-OH of the sugar ring; it was active against the 2′-*O*-methyl derivative of cytidine, but not arabinosycytosine.

The N^4-and 5′-*O*-alkyl non-substrate analogues competitively inhibited deamination of deoxycytidine and arabinosylcytosine, the most inhibitory being 5′-*O*-methylarabinosylcytosine.

The alpha anomer of 5-ethyldeoxycytidine, the 2,2′-anhydro derivative of cytidine, and the 3′-*O*-alkyl derivatives were neither substrates nor inhibitors.

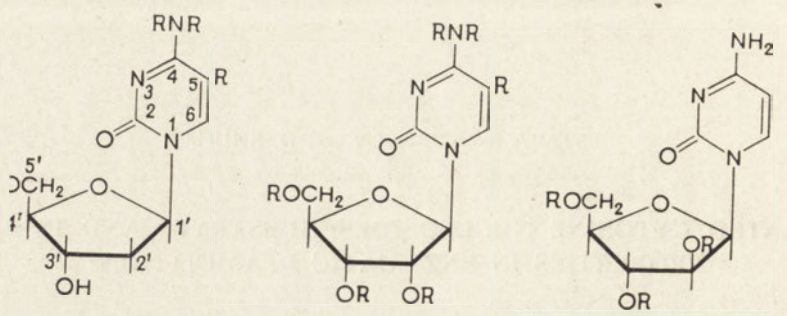
The presence of cytidine deaminase was demonstrated in both granulocytes and lymphocytes from human peripheral blood. The specificity of this enzyme differed significantly from that of the bacterial enzyme, a finding of some relevance in relation to the frequently encountered intracellular deamination of therapeutically active arabinosylcytosine to the inactive arabinosyluracil.

Intracellular enzymatic deamination of araC[1], a potent drug against acute leukemia, to the therapeutically inactive araU (Camiener & Smith, 1965; Creasey *et al.*, 1966), is responsible to a considerable extent for the reduction in clinical efficiency of the drug. Considerable effort has therefore been devoted to studies on the nature of the enzyme responsible for this reaction, i.e. cytosine nucleoside aminohydrolase

* This investigation was carried out as project 09.3.1. of the Polish Academy of Sciences, and profited from the partial support of the Agricultural Research Service, U.S. Department of Agriculture.

[1] Abbreviations used: araC, 1-β-D-arabinofuranosylcytosine; araU, 1-β-D-arabinofuranosyluracil; Cyd, cytidine; dCyd, 2′-deoxycytidine.

(EC 3.5.4.5), and the preparation of inhibitors of this reaction, or of analogues resistant to deamination; the importance of this problem is further underlined by the established activity of araC against some DNA viruses (for review, see Shugar, 1974).



1-β-D-2'-Deoxyribofuranosylcytosine

1-β-D-Ribofuranosylcytosine

1-β-D-Arabinofuranosylcytosine

R=H or CH_3 or C_2H_5

Scheme 1

The preparation in these laboratories of a variety of alkylated cytosine nucleosides, schematically represented in Scheme 1, prompted us to undertake an investigation of the substrate and/or inhibitory properties of a number of these analogues in a cytidine deaminase system. The source of enzyme employed was a cell-free extract of *Salmonella typhimurium* LT2, which possessed a metabolically derepressed cytidine deaminase system.

MATERIALS AND METHODS

Nucleosides and nucleotides: N_3-Methylcytidine was obtained from Sigma (St. Louis, Mo., U. S. A.). All the other alkylated cytosine nucleoside derivatives were prepared as elsewhere described (Darżynkiewicz *et al.*, 1972; Kuśmierek *et al.*, 1973; Kulikowski & Shugar, 1974; Darżynkiewicz & Shugar, 1974). The remaining nucleosides, and nucleotides were A grade from commercial sources (Sigma, St. Louis, Mo., U. S. A.; Calbiochem, Zurich, Switzerland), and checked by chromatography (see Table 1).

Growth procedures: An overnight inoculum of *S. typhimurium* LT2 in a glycerol--mineral salts medium (Vogel & Bonner, 1956) was diluted about 50× with the same medium and supplemented with 0.1% of uridine or cytidine as the inducer. Incubation was then carried out at 37°C with shaking until the cells had undergone 5 - 6 divisions. The cells were collected by centrifugation, washed with 0.9% saline, and used immediately or stored at -20°C.

Toluenized cells: Washed cells were suspended in 1/5 the original incubation volume of 0.1 M-Tris-HCl buffer, pH 7.5, and $MgCl_2$ added to a concentration of 1 mM. Toluene was then added to a concentration of 1% and the cell suspension

gently shaken at room temperature for 15 min. The cells were collected by centrifugation, resuspended in the same buffer and, as such, used directly for enzyme assays.

Cell-free extracts: Extracts were prepared by ultrasonic treatment of the washed cells, using an MSE 100 Watt ultrasonic disintegrator. Sonication was carried out 3 times for 1-minute intervals, with 1-minute breaks, on cells suspended in 1/20 the original volume of 0.1 M-Tris-HCl buffer, pH 7.5, 1 mM-$MgCl_2$ and 0.6 mM-2-mercaptoethanol. Following centrifugation for 30 min at 18 000 *g* and 4°C, the supernatant was employed as a source of enzyme.

Enzyme assays: Deaminase activity was monitored spectrophotometrically as described by Wang (1955), with slight modifications. In acid medium the u. v. absorption spectra of cytosine nucleosides (including *O'*-alkyl analogues) differ from the corresponding uracil nucleosides at 290 nm by $\Delta\varepsilon = -10.1 \times 10^3$, and for the analogous 5-alkyl derivatives of these nucleosides this difference is -8.7×10^3 at 290 nm and -7.0×10^3 at 300 nm.

Unless otherwise indicated, incubation mixtures contained, in a final volume of 0.2 ml, 20 µmoles Tris-HCl buffer, pH 7.5, 0.2 µmole $MgCl_2$ and appropriate concentrations of nucleosides and bacterial extract. The reaction (at 37°C) was terminated by addition of 0.8 ml of 0.5 M-$HClO_4$ at 0°C. The reference blanks contained all the constituents, with the exception that substrate was added to the cold mixture immediately prior to acidification. When comparing the rates of deamination of various substrates, the reactions were terminated at a time interval which was on the linear portion of the plot of percent substrate hydrolysis *versus* time.

U. v. absorption measurements were carried out on a manual Zeiss (Jena, D. D. R.) VSU-2 instrument or on a Unicam SP-800 recording spectrophotometer, using 10-mm pathlength semi-micro cuvettes.

Chromatography: In some instances the reaction was followed by paper (Whatman No. 1) or cellulose (thin-layer, Merck, Darmstadt, G. F. R.) chromatography. In such cases reactions were terminated by immersion of the reaction mixture in a boiling water bath for 1 - 2 min; following centrifugation and washing of the pellet, the pooled supernatant was concentrated and spotted on the chromatograms.

Spots were located under a dark u. v. lamp, eluted with water, and the spectra of the eluates determined at different pH values. Compounds were identified on the basis of R_F values and spectra relative to those for standard substances (Table 1).

Kinetic constants: Michaelis-Menten constants (K_m) and maximal velocities (V_{max}) were obtained graphically from double-reciprocal plots of substrate concentration *versus* initial velocity. In those instances when reactions were carried out in the presence of analogues, similar graphical procedures were employed to establish the nature of the inhibitory effect and the associated K_i value.

Protein estimation: The colorimetric method of Lowry *et al.* (1951) and/or the spectrophotometric method of Groves *et al.* (1968) were applied, using bovine serum albumin as standard.

Inorganic phosphate (P_i) was determined by the spectrophotometric method of Chen *et al.* (1956).

Table 1

Chromatographic mobilities of cytosine nucleosides, their deamination products, and the corresponding pyrimidine bases

Ascending chromatography with Whatman paper No. 1 and the following solvent systems (all ratios v/v): (*A*) ethyl acetate - formic acid - water (12:1:7, upper phase); (*B*) ethyl acetate - formic acid - - water (7:1.5:1.5); (*C*) *n*-butanol - 5% NH_4OH (3 : 1); (*D*) water-saturated *sec.*-butanol.

Compound	R_F value			
	A	*B*	*C*	*D*
Uracil	0.29	0.77	0.15	0.59
5-Methyluracil	0.48	0.80	0.38	0.75
Cytosine	0.01	0.42	0.23	0.37
Cytidine	0.005	0.27	0.10	0.34
Uridine	0.05	0.39	0.02	0.47
5-Methylcytidine	0.01	0.33	0.16	0.38
5-Methyluridine	0.15	0.52	0.17	0.58
5-Ethylcytidine	0.02	0.49	0.27	0.48
5-Ethyluridine	—	0.69	—	0.65
3-Methylcytidine	0.01	0.36	0.31	0.21
3-Methyluridine	0.30	0.55	0.42	0.69
2′-Deoxycytidine	0.01	0.60	0.24	0.42
2′-Deoxyuridine	0.17	0.71	0.13	0.59
5-Methyl-2′-deoxycytidine	0.01	0.67	—	0.48
5-Methyl-2′-deoxyuridine	0.27	—	0.33	0.71
5-Ethyl-2′-deoxycytidine	0.03	—	0.44	0.59
5-Ethyl-2′-deoxyuridine	—	—	—	0.83
2′-*O*-Methylcytidine	0.02	—	0.29	0.48
2′-*O*-Methyluridine	—	0.67	0.12	0.66
1-β-D-Arabinosylcytosine	0.005	0.32	0.14	0.37
1-β-D-Arabinosyluracil	0.36	—	0.08	0.58

RESULTS

Deamination of nucleosides. Table 2 presents the results for the relative rates of deamination of the various cytosine nucleosides and their analogues. It will be noted that the best substrate was dCyd, followed in turn by Cyd and araC. Substitution of an alkyl group at C-5 of the pyrimidine ring did not appreciably reduce substrate susceptibility, e. g. introduction of a 5-methyl group reduced the rate of deamination to 0.6 - 0.8 of that of the unsubstituted nucleoside. However, replacement of a 5-methyl by a 5-ethyl led to a further, and even greater, decrease in susceptibility to deamination.

Amongst the *O*′-alkyl analogues, only 2′-*O*-methyl-Cyd proved a good substrate, its rate of deamination being almost one-half that for the parent Cyd. It is, however, of interest that replacement of the 2′-*O*-methyl by a 2′-*O*-ethyl almost completely abolished susceptibility to deamination, most probably as a result of steric effects. It is also worth noting that the corresponding 2′-*O*-methyl-araC, although highly resistant, was deaminated at a detectable rate.

Table 2

Relative rates of deamination of cytosine nucleosides by the cell-free extract of S. typhimurium

Conditions as described in Methods

Compound	Relative activity	Compound	Relative activity
2′-Deoxycytidine	1.0	Cytidine	0.5
5-Methyl-	0.7	5-Methyl-	0.38
5-Ethyl- (β anomer)	0.23	5-Ethyl-	0.10
5-Ethyl- (α anomer)	0.0	3-Methyl-	0.0
N^4-Methyl-	0.0	N^4-Methyl-5-ethyl-	0.0
N^4,5-Dimethyl-	0.0	N^4,5-Diethyl-	0.0
N^4,N^4,5-Trimethyl-	0.0	2′-*O*-Methyl-	0.22
araC	0.35	2′-*O*-Ethyl-	<0.01
2′-*O*-Methyl-	<0.01	3′-*O*-Methyl-	0.0
3′-*O*-Methyl-	0.0	5′-*O*-Methyl-	0.0
5′-*O*-Methyl-	0.0	5′-*O*-Ethyl-	0.0
5′-*O*-Ethyl-	0.0	2′,3′-Di-*O*-methyl-	0.0
2,2′-Anhydro-	0.0	2′,3′,5′-Tri-*O*-methyl-	0.0

The apparent kinetic data (Table 3) are in reasonably good agreement with the data in Table 2, both as regards the affinity properties (K_m values) and maximal velocities (V_{max}).

The relative activities of all compounds tested, with the exception of araC, were similar when tested with cell-free extracts (Table 2), with intact cells and with permeable (i. e. toluenized) cells. The rate of deamination of araC by intact cells was less than 0.1 that for deamination of dCyd. This value increased to 0.25 - 0.40 with permeable cells. The difference may possibly be due to some inhibitory step in the transport of araC through the cell membrane of intact cells, although it is tacitly assumed that araC is as readily transported through the membranes of various cells as cytidine or deoxycytidine.

Table 3

Apparent kinetic constants for deamination of cytosine nucleosides

Nucleoside	K_m (μmoles)	V_{max} (μmoles × mg protein^{-1} × min^{-1})
2′-Deoxycytidine	86	1.01
5-Methyl-	120	0.52
5-Ethyl-	436	0.17
Cytidine	184	0.49
5-Methyl-	246	0.32
5-Ethyl-	1460	0.10
2′-*O*-Methyl-	555	0.19
araC	290	0.38

Inhibitory properties of analogues. The various analogues were tested for their ability to inhibit the deamination reaction, using dCyd and araC as substrates, the former because it is the best substrate, and the latter because of its therapeutic interest. It was found that deamination of both of these substrates was inhibited by all the analogues which were themselves substrates. The results with the non-substrate analogues are summarized in Table 4, from which it will be noted that the alpha anomer of 5-ethyl-2′-dCyd, and the 2,2′-anhydro- and 3′-*O*-methyl derivatives, exhibit little or no inhibition. By contrast, analogues alkylated on the exocyclic amino group exhibit some inhibition, which is appreciably enhanced by introduction of an additional alkyl at N^4 or C-5. Both the 2′-*O*- and 5′-*O*-alkyl derivatives inhibit significantly, 5′-*O*-methyl-araC being the most inhibitory of all the analogues examined.

Table 4

Inhibitory effects of non-substrate analogues on deamination of dCyd and araC

Ratio of analogue to substrate concentration 1:1. Inhibition (in %) expressed as follows: —, <5; ·, 5 - 15; +, 15 - 30; ++, >30.

Compound	dCyd	araC
5-Ethyl-2′-dCyd	—	—
N^4-Methyl-2′-dCyd	·	+
N^4,5-Dimethyl-2′-dCyd	+	++
N^4-Ethyl-5-methyl-2′-dCyd	+	++
N^4,N^4,5-Trimethyl-2′-dCyd	+	++
N^4,5-Diethyl-Cyd	·	+
2′-*O*-Ethyl-Cyd	·	+
3′-*O*-Methyl-Cyd	—	—
5′-*O*-Methyl-Cyd	·	+
5′-*O*-Ethyl-Cyd	+	·
2′,3′-Di-*O*-methyl-Cyd	—	—
2′,3′,5′-Tri-*O*-methyl-Cyd	—	—
2′-*O*-Methyl-araC	·	+
3′-*O*-Methyl-araC	—	—
5′-*O*-Methyl-araC	++	++
5′-*O*-Ethyl-araC	·	+
2,2′-Anhydro-araC	—	—
2,2′-Anhydro-5′-*O*-methyl-araC	—	—

The specific inhibitory effect of 5′-*O*-methyl-araC is further testified to by the fact that, under similar conditions, the deamination reaction was virtually unaffected by such nucleoside-5′-phosphates as CMP, dCMP, dTMP, UMP, dUMP, araCMP. Under the experimental conditions employed, dephosphorylation of the foregoing nucleotides proceeded to only a minimal extent (<10%), checked by estimations of inorganic phosphate.

The results of kinetic studies, illustrated in Figs. 1 and 2, indicate that the inhibitory effects exhibited by the non-substrate analogues are competitive in nature. The calculated K_i values are listed in Table 5.

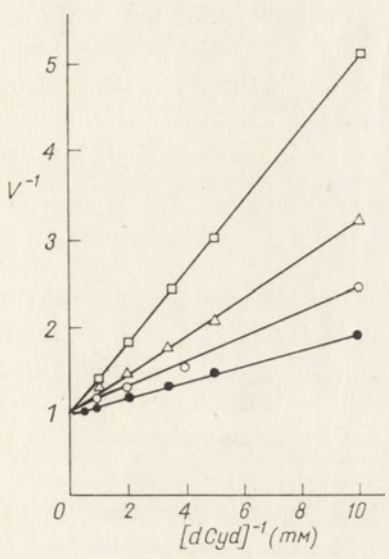


Fig. 1.

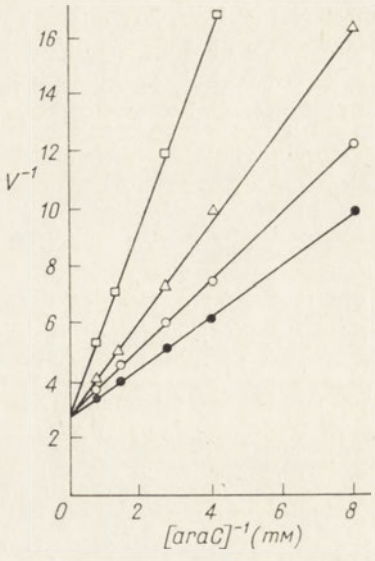


Fig. 2.

Fig. 1. Double-reciprocal plot of the activity of cytosine nucleoside deaminase *versus* deoxycytidine concentration in the absence (●) and presence of the non-substrate analogues: 0.6 мм-N^4-methyldeoxycytidine (○); 0.6 мм-N^4-dimethyldeoxycytidine (△); and 0.75 мм-5′-*O*-methyl-araC (□). *V* is expressed in μmoles of deaminated dCyd × mg protein^{-1} × min^{-1}.

Fig. 2. Double-reciprocal plot of the activity of cytosine nucleoside deaminase *versus* araC concentration in the absence (●) and presence of the non-substrate analogues: 1 мм-2′-*O*-methyl-araC (○); 1 мм-N^4,5-dimethyldeoxycytidine (△); and 1.25 мм-5′-*O*-methyl-araC (□). *V* is expressed in μmoles of deaminated araC × mg protein^{-1} × min^{-1}.

Table 5

K_i *values of the non-substrate analogues* (*in* мм)

Analogue	Substrate	
	dCyd	araC
5′-*O*-Methyl-araC	0.63	0.27
2′-*O*-Methyl-araC	—	4.8
N^4-Methyl-dCyd	8.1	—
N^4,5-Dimethyl-dCyd	2.6	1.3

Trials with a mammalian deaminase. Bearing in mind the therapeutic significance of araC, it becomes of obvious interest to extend specificity studies to mammalian deaminase systems. Some preliminary results were obtained on normal human peripheral blood, which was fractionated into plasma, red cells and granulocytes and lymphocytes. No deaminase activity was detectable either in the plasma or the red cells. Both granulocytes and lymphocytes, however, exhibited appreciable activity. In this case cytidine was the most active substrate, followed by deoxycytidine and araC, both of which were deaminated at about 70% of the rate for cytidine. As

for the bacterial enzyme, 2′-*O*-methylcytidine was a substrate, but its susceptibility relative to cytidine was only one-half that with the bacterial enzyme. More significant was the fact that, in contrast to the bacterial enzyme, 5′-*O*-methylcytidine exhibited low susceptibility to deamination, and this was further accentuated in the case of 5′-*O*-methyl-araC, which underwent deamination at about 15% the rate for araC itself.

DISCUSSION

The observed relative rates of deamination of the common substrates, deoxycytidine > 5-methyldeoxycytidine > cytidine > araC (Table 2) are in the same order as those reported with other bacterial enzymes, including a purified cytidine deaminase preparation from *E. coli* (Hosono & Kuno, 1973). In the latter instance the measured K_m values for deoxycytidine and cytidine are similar to those obtained in the present study (Table 3).

The influence of 5-methyl and 5-ethyl substituents is practically identical for both Cyd and dCyd, the relative decreases in rates being the same for both nucleosides. This decrease in rate resulting from the introduction of a 5-methyl has previously been observed by others (Cohen & Barner, 1957; Wempen *et al.*, 1961; Camiener, 1967; Wisdom & Orsi, 1969; Hosono & Kuno, 1973). The further decrease on replacement of a 5-methyl by a 5-ethyl is undoubtedly due to simple steric hindrance. This is supported by the fact that, with both the *E. coli* (Cohen & Barner, 1957) and the human liver (Camiener, 1967) enzymes, introduction of a 5-halogen substituent decreases the rate of deamination in the order 5-chloro > 5-bromo > 5-iodo, i. e. with increasing van der Waals radius of the halogen substituent, the susceptibility to deamination decreases.

Some differences of opinion exist as to the effects of modification of the exocyclic amino group. It has been reported by Wempen *et al.* (1961), Trimble & Maley (1971) and Cohen & Wolfenden (1971) that analogues with a methylated amino group still show susceptibility to deamination, albeit at a considerably reduced rate. Our own findings indicate that an unmodified amino group is essential, in agreement with the results of Camiener (1967) and Dollinger *et al.* (1967).

As regards the substrate or inhibitory properties of the *O′*-alkyl derivatives, it is pertinent to note that the conformations of the sugar rings are affected only to a minor extent by the introduction of *O′*-alkyl group (Hruska *et al.*, 1973; Remin & Shugar, 1973), so that the influence of such *O′*-alkylation is probably the result of simple steric effects.

Etherification of the 3′-OH or the 5′-OH leads to a virtually complete loss of susceptibility to deamination. It would be of considerable interest in this connection to examine the reactivity of the 3′- and 5′-deoxy derivatives of araC, as well as 3′-deoxycytidine and 5′-deoxycytidine.

Since alkylation of 3′-OH results in the loss of both substrate and inhibitory activities of the compounds, the integrity of this group may be decisive for the formation of the enzyme-nucleoside complex.

If we consider the effects of substitution in the sugar ring, then, for the 2′- position, our results (Table 2) demonstrate the following sequence of decreasing susceptibilities to the bacterial enzyme:

$$2'\text{-}H_{trans} > 2'\text{-}OH_{trans} > 2'\text{-}OH_{cis} > 2'\text{-}OCH_{3\,trans} \gg 2'\text{-}OCH_{3\,cis}$$

where *cis* and *trans* refer to configurations relative to the 5′-CH_2OH group, e. g. the 2′-OH in araC is *cis* with respect to the 5′-CH_2OH. A similar preference for deoxycytidine is shown by the enzyme from *E. coli* (Cohen & Barner, 1957; Wempen *et al.*, 1961; Trimble & Maley, 1971; Hosono & Kuno, 1973). By contrast, the enzyme from all mammalian sources hitherto investigated, including our own findings with the enzyme from serum lymphocytes and granulocytes, exhibit a definite preference for cytidine, relative to deoxycytidine, pointing to a marked difference in interaction with the substrate between the two classes of enzyme.

The results with the enzyme from the white cells of human peripheral blood, although of a preliminary nature, call for some additional comment. The difference in specificity as compared to the bacterial enzyme, is further accentuated by the fact that 5′-*O*-methyl-araC, which is virtually resistant to the bacterial enzyme, is a reasonably good substrate for the enzyme from white cells. Equally significant is the poor susceptibility of the 5-ethyl derivatives of cytidine and deoxycytidine to the white cell enzyme; the resistance to deamination of 5-ethyldeoxycytidine may explain the lack of expected antiviral activity of this analogue in a system where 5-ethyldeoxyuridine exhibited significant activity (De Clercq & Shugar, 1975).

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ALKILOWANE NUKLEOZYDY CYTOZYNY: WŁASNOŚCI SUBSTRATOWE I INHIBITOROWE W REAKCJI ENZYMATYCZNEJ DEZAMINACJI

Streszczenie

Dezaminaza nukleozydów cytozyny (EC 3.5.4.5) z *Salmonella typhimurium* LT2 katalizuje dezaminację analogów rybo-, dezoksyrybo- i arabinozylocytozyny alkilowanych przy C-5 pierścienia pirymidynowego. Enzym nie działa natomiast na pochodne alkilowane przy N_3 lub przy egzocyklicznej grupie aminowej, ani też na analogi posiadające wiązania eterowe przy grupach 3′-OH i 5′-OH cukru.

Analogi N^4- i 5′-*O*-alkilowane, nie będące substratami, hamują kompetycyjnie dezaminację dezoksycytydyny i arabinozylocytozyny; najsilniejszym inhibitorem spośród badanych związków okazał się 5′-*O*-metylo-araC.

Alfa anomer 5-etylodezoksycytydyny, 2,2′-bezwodnik cytydyny i 3′-*O*-alkilowane pochodne nie są ani substratami ani inhibitorami reakcji.

Stwierdzono obecność dezaminazy cytydynowej w granulocytach i limfocytach obwodowej krwi ludzkiej. Specyficzność tego enzymu różni się w sposób istotny od specyficzności enzymu z bakterii, co ma znaczenie w związku z dezaminacją w organizmie arabinozylocytozyny, skutecznego leku przy białaczkach, do terapeutycznie nieaktywnego arabinozylouracylu.

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