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UTILIZATION OF [^{14}C]OROTATE IN THE BIOSYNTHESIS OF PYRIMIDINES IN *HELIX POMATIA* AND *CELERIO EUPHORBIAE*

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1. After injection of radioactive orotate to *Celerio euphorbiae* pupae, active UMP and CMP were found in RNA isolated from the fat body and muscle. 2. In RNA from *Helix pomatia* hepatopancreas, radioactive UMP and CMP were found after administration of labelled orotate, aspartate or bicarbonate. 3. The presence of aspartate carbamoyltransferase in snail hepatopancreas was demonstrated. 4. In the two invertebrates studied the biosynthesis of the pyrimidine ring follows probably the same pathway as in vertebrates.

There are no data in the literature which would indicate that in invertebrate animals the biosynthesis of pyrimidine follows the same pathway as in vertebrates, i.e. from carbamoylphosphate and aspartate *via* orotate. The question is also open whether or not the invertebrates are able to synthesize the necessary carbamoylphosphate. Although the investigations of Bishop & Campbell suggested the possibility of carbamoylphosphate synthesis in the tissues of *Otala lactea* [5] and *Lumbricus terrestris* [1, 2], the isotopic studies of Bricteux-Grégoire & Florkin [4] did not demonstrate the synthesis of this compound in the hepatopancreas of *Helix pomatia*.

In the present work an attempt was made to ascertain whether orotate can serve as precursor for synthesis of pyrimidine nucleotides in those uricotelic invertebrates in which so far the presence of carbamoylphosphate synthase (EC 2.7.2.5) has not been demonstrated.

The experiments were carried out on the snail *Helix pomatia* and on the pupa of *Celerio euphorbiae* moth. As demonstrated previously [10], both the muscle and hepatopancreas of *H. pomatia* possess ornithine carbamoyltransferase (EC 2.1.3.3) and a very active arginase (EC 3.5.3.1), whereas argininosuccinate synthetase (EC 6.3.4.5) has not been found. No enzymes of the ornithine cycle could be found in *C. euphorbiae* except a weak arginase activity [11]. Neither of the animals studied was able to synthesize urea through the ornithine cycle and the arginase found in their tissues most probably catalysed the degradation of arginine derived from phosphoarginine or proteins.

MATERIALS AND METHODS

Adult snails *Helix pomatia* during the feeding period and *Celerio euphorbiae* pupae during diapause, were studied. In each experiment the labelled compound, diluted to 100 μc/ml., was injected into the hepatopancreas of two snails and into the fat body of five pupae in doses, respectively, of 2 and 3 μc per animal. At 10 or 20 hr. after the injection, snail hepatopancreas and fat body and muscle of the pupa were isolated and homogenized in water at 0° for 1 min. After centrifuging the supernatant fluid was mixed with an equal volume of 90 % phenol, and RNA was isolated according to Kirby [7]. The aqueous solution of RNA was concentrated to a small volume under vacuum and incubated for 16 hr. at 37° with an equal volume of 0.6 N-KOH. The RNA hydrolysate was acidified with perchloric acid to pH 1 - 2. The precipitated sediment was centrifuged, washed twice and then discarded. The supernatant and the wash fluids were pooled, adjusted to pH 7, applied to the Dowex 1 X8 (formate form, 200 - 400 mesh) column (0.5×25 cm.), and the nucleotides were eluted by a formic acid gradient. The mixing vessel contained 25 ml. of water and the reservoir 45 ml. of 0.5 N-formic acid with subsequent addition of 105 ml. of 4.0 N-formic acid; 5 ml. portions of the eluates were collected. Extinctions of the separate eluates were measured in a Beckman type spectrophotometer SF 4 at 260 mμ. Fractions containing the same nucleotide were pooled, then every fraction was divided into two parts. Each part was evaporated until dry under reduced pressure, then washed several times with water to remove formic acid. One sample served for nucleotide determination; the dry residue was dissolved in 3 ml. of 0.01 N-NaOH and the absorption spectrum was studied. The amount of the nucleotide was calculated using the molar extinction coefficient. In the second sample the radioactivity was measured in the scintillation counter; the dry residue was dissolved in 0.2 ml. of 0.2 N-NaOH and transferred to a counting vial which contained 10 ml. of a dioxan-based scintillation mixture prepared according to Bray [3]. The efficiency of this counting system was about 30%.

Aspartate carbamoyltransferase (carbamoylphosphate : L-aspartate carbamoyltransferase, EC 2.1.3.2) in snail hepatopancreas was assayed *in vitro* by the formation of ureidosuccinate. The assays were carried out according to Lindsay, Nakagawa & Cohen [9]; the incubation mixture contained in 3.2 ml.: 50 μmoles of tris buffer, pH 9.2; 20 μmoles of sodium [U-^{14}C]aspartate, 4 μc; 50 μmoles of carbamoylphosphate; and 1 ml. of the enzyme (105 000 *g* at 30 min. supernatant solution of hepatopancreas homogenate in 0.25 M-sucrose). After 1 hr. of incubation at 37° the reaction was stopped by adding perchloric acid to a final concentration of 0.5 N. The perchloric acid was removed from the deproteinized material by adding KOH; after centrifuging the supernatant was adjusted to pH 7 and applied to the column (0.5×25 cm.) of Dowex 1 X8 (Cl^- form) 200 - 400 mesh. Elution was with a gradient of hydrochloric acid. The mixing vessel contained 25 ml. of water and the reservoir 100 ml. of 0.05 N-HCl. Three-milliliter fractions were collected, and in each fraction the extinction at 260 mμ was read and radioactivity measured in the scintillation counter. The radioactive compound which was eluted in tubes

27 - 29 similarly as the authentic ureidosuccinate, was also identified as ureidosuccinate by paper chromatography.

Special reagents. [6-^{14}C]Orotic acid (33 mc/m-mole), Radiochemical Centre, Amersham, England. [U-^{14}C]Aspartic acid (8.9 mc/m-mole) and sodium [^{14}C]-bicarbonate (3 mc/m-mole), UVVVR, Prague, Czechoslovakia.

Carbamoylphosphate, dilithium salt of 65 - 75% purity, was prepared from potassium cyanate and potassium dihydrogen phosphate after Jones, Spector & Lipmann [6]. L-Ureidosuccinic acid was synthesized from L-aspartic acid and potassium cyanate according to the method described by Korn [8]. From the reaction mixture L-ureidosuccinic acid was isolated by Dowex 2 column chromatography and its purity was checked by ascending paper chromatography on Whatman no. 1 paper. One spot only was obtained in each of the following solvent systems [9]: butanol - acetic acid - water (80 : 16 : 40, by vol.), R_F 0.43; propanol - water (10 : 3, v/v), R_F 0.27; ethyl acetate - methanol - formic acid - water (6 : 6 : 3 : 5, by vol.), R_F 0.77.

RESULTS AND DISCUSSION

After administration of ^{14}C-labelled orotate to the animals studied, the radioactivity was recovered both in RNA of snail hepatopancreas and in RNA isolated from the fat body and muscle of *C. euphorbiae* pupa (Table 1), the incorporation being greater 20 hr. than 10 hr. after the injection. Both in the snail and in pupa

Table 1

Incorporation of [6-^{14}C]*orotate into mononucleotides of RNA from hepatopancreas of* Helix pomatia *and fat body and muscle of* Celerio euphorbiae *pupa*

For each series of experiments 2 snails and 5 pupae were used. The snails were injected with 3 μc and pupae with 2 μc of [6-^{14}C]orotate. After indicated time intervals, RNA was isolated and after alkaline hydrolysis the nucleotides were separated on a Dowex 1 X8 column (for details see Methods).

Nucleotide isolated	Time after injection (hr.)	*Helix pomatia* (hepatopancreas)		*Celerio euphorbiae* (fat body and muscle)	
		Nucleotide		Nucleotide	
		(μmole)	(counts/min./μmole)	(μmole)	(counts/min./μmole)
CMP	10	0.420	157	0.236	1 710
	20	0.496	554	0.229	6 670
AMP	10	0.447	0	0.209	0
	20	0.423	260	0.198	0
GMP	10	0.584	0	0.250	0
	20	0.596	96	0.243	282
UMP	10	0.570	610	0.231	12 370
	20	0.562	1515	0.210	30 120

uridylic acid (UMP) possessed the highest specific activity; in snail hepatopancreas after 10 hr. it was 4 times as high, and after 20 hr. 2.5 times as high, as in cytidylic acid (CMP), and in pupa, respectively, 7 and 4.5 times as high. It can be supposed that in both those animals, similarly as in vertebrates and bacteria, UMP is a precursor in the synthesis of CMP.

The extent of incorporation of orotate into pyrimidines in snail hepatopancreas differed from that in pupa tissues. In the pupa the specific activity of UMP, both after 10 and 20 hr., was 20 times as high as in the snail, and the activity of CMP 12 times as high.

At 10 hr. after orotate injection no radioactivity was found in purine nucleotides, and at 20 hr. a slight activity was found only in GMP of the pupa and in GMP and AMP of the snail.

Utilization of earlier precursors of pyrimidine biosynthesis was studied in the snail. At 24 hr. after the administration of [U-^{14}C]aspartate, UMP from hepatopancreas RNA was found to possess the highest specific radioactivity, whereas the activity observed in CMP corresponded only to half of the value for UMP (Table 2). The radioactivity of purines was also high and, as aspartate has not been shown to be a direct contributor of carbon for purine biosynthesis, this activity was probably due to the incorporation of the ^{14}C-labelled aspartate degradation products. When labelled bicarbonate was administered, after 24 hr. the same radioactivity was found in pyrimidine and purine nucleotides of RNA (Table 2).

Table 2

Specific activities of RNA mononucleotides from hepatopancreas of Helix pomatia, *after injection of sodium* [^{14}C]*bicarbonate and* [U-^{14}C]-*aspartate*

For each series of experiments 3 snails in the feeding period were used. [^{14}C]$NaHCO_3$, 5 μc, was injected into the hepatopancreas at the beginning of the experiment and 2 μc after 10 hr. [U-^{14}C]Aspartate, 3μc, was administered in a single dose. After 24 hr. RNA was isolated and after alkaline hydrolysis the nucleotides were separated on a Dowex 1 X8 column (for details see Methods).

Nucleotide isolated	Sodium [^{14}C]bicarbonate		[U-^{14}C]Aspartate	
	Nucleotide		Nucleotide	
	(μmole)	(counts/min./μmole)	(μmole)	(counts/min./μmole)
CMP	0.893	118	1.010	141
AMP	0.767	106	0.923	81
GMP	0.740	157	0.986	161
UMP	0.812	181	1.074	319

In experiments *in vitro*, formation of ureidosuccinate, which is a precursor of orotate, was studied in the snail. It was found that during incubation of the 105 000 *g* supernatant from hepatopancreas homogenate, with carbamoylphosphate and labelled aspartate, radioactive ureidosuccinate was formed (Fig. 1). It was identified by comparison with authentic ureidosuccinic acid, having the same elution

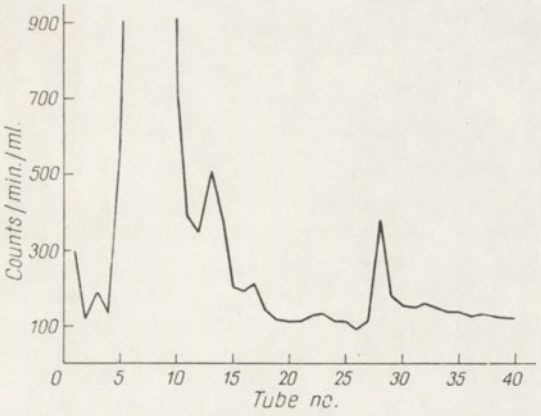

Fig. 1. Separation by Dowex 1 X8 column chromatography of radioactive compounds formed after incubation of the 105 000 *g* supernatant from the homogenate of *Helix pomatia* hepatopancreas, with [U-^{14}C]aspartate and carbamoylphosphate. In tubes 27 - 29 the presence of ureidosuccinic acid was confirmed by paper chromatography

characteristics on a Dowex 1 X8 column and identical R_F values on paper chromatography.

The presented results permit to suggest that in both invertebrates studied the synthesis of the pyrimidine ring may take place *via* orotate, similarly as in vertebrates.

REFERENCES

[1] Bishop S. H. & Campbell J. W. - *Science* **142**, 1583, 1963.
[2] Bishop S. H. & Campbell J. W. - *Comp. Biochem. Physiol.* **15**, 51, 1965.
[3] Bray G. A. - *Anal. Biochem.* **1**, 279, 1960.
[4] Bricteux-Grégoire S. & Florkin M. - *Comp. Biochem. Physiol.* **12**, 55, 1965.
[5] Campbell J. W. & Bishop S. H. - *Biochim. Biophys. Acta* **77**, 149, 1963.
[6] Jones M. E., Spector L. & Lipmann F. - *J. Amer. Chem. Soc.* **77**, 819, 1955.
[7] Kirby K. S. - *Biochem. J.* **64**, 405, 1956.
[8] Korn E. D. in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. IV, p. 615. Academic Press, New York 1957.
[9] Lindsay R. H., Nakagawa H. & Cohen P. P. - *J. Biol. Chem.* **239**, 2239, 1964.
[10] Porembska Z. & Heller J. - *Acta Biochim. Polon.* **9**, 385, 1962.
[11] Porembska Z. & Mochnacka I. - *Acta Biochim Polon.* **11**, 113, 1964.

WYKORZYSTANIE [^{14}C]OROTANU W BIOSYNTEZIE PIRYMIDYN U *HELIX POMATIA* I *CELERIO EUPHORBIAE*

Streszczenie

1. Po injekcji radioaktywnego orotanu poczwarkom *Celerio euphorbiae* w RNA izolowanym z ciała tłuszczowego i mięśnia stwierdzano aktywny UMP i CMP.

2. W RNA z hepatopancreas ślimaka odnajdowano aktywny UMP i CMP po injekcji znaczonego orotanu, asparaginianu i dwuwęglanu.

3. W hepatopancreas ślimaka wykazano obecność transkarbamylazy asparaginowej.

4. U badanych bezkręgowców synteza pierścienia pirymidynowego przebiega prawdopodobnie identycznie jak u kręgowców.

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THE EFFECT OF NITROGEN MUSTARDS ON GLUTAMATE METABOLISM IN RAT LIVER MITOCHONDRIA AND EHRLICH ASCITES CELLS

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1. Mannitol nitrogen mustard given to rats in LD_{50} dose did not affect the activity of NAD glycohydrolase, glutamate dehydrogenase and aspartate - 2-oxoglutarate aminotransferase in liver mitochondria, although it decreased the leucocyte count by 50 - 70%. 2. In Ehrlich ascites cells from nitrogen mustard-treated mice the increase in NAD glycohydrolase and the decrease in concentration of pyridine nucleotides was accompanied by an eightfold decrease in the activity of glutamate dehydrogenase. The activity of aspartate - 2-oxoglutarate aminotransferase was unaffected. 3. The results are confronted with the changes in purine components of RNA.

Nitrogen mustard and its derivatives, widely used as therapeutics in carcinoma, are generally believed to act as alkylating agents on nucleic acids and proteins, primarily on the nucleic acid nitrogen [18, 13]. In addition to the evidence indicating alkylation of nucleic acids *in vivo* and the influence of nitrogen mustards on various enzymic activities [19], data have been accumulated proving the decrease in concentration of pyridine nucleotides in neoplasma of the animals treated with nitrogen mustards [18, 1]. Thus the decrease in the rate of glycolysis observed in neoplastic tissues [6] may be due both to the decreased activities of glycolytic enzymes and the lowered content of coenzymes required. In 1962 Green & Bodansky [7] showed that a 50 - 70% decrease in NAD content in Ehrlich ascites cells from mice treated with nitrogen mustard was caused by a 4 - 5-fold activation of NAD glycohydrolase (EC 3.2.2.5). In consequence the rate of lactate formation was markedly reduced.

The presented experiments were performed on liver mitochondria of healthy rats treated with mannitol and propylenephosphodiamide derivatives of nitrogen mustard, drugs of established clinical value, to find whether the action of these compounds on NAD concentration *via* the NAD glycohydrolase system constitutes a general mechanism of the nitrogen mustard activity toward the NAD-dependent enzymes. These enzymes were exemplified by glutamate dehydrogenase (L-glutamate : NAD oxidoreductase (deaminating), EC 1.4.1.2). The alternative route of glutamate metabolism, i.e. transamination by the L-aspartate : 2-oxoglu-

tarate aminotransferase (EC 2.6.1.1) was also studied to show the possible effect of nitrogen mustard on the equilibrium of both systems metabolizing glutamate.

Parallel experiments were carried out on Ehrlich ascites cells.

MATERIALS AND METHODS

Reagents. Nitrogen mustards: dichlorodiethylmethylamine hydrochloride,HN2 (Nitrogranulogen, Polfa, Krak. Zakł. Farm., Poland); 1,6-bis(β-chloroethylamine)--1,6-deoxymannitol dichloride (Degranol-chinoin, Medimpex, Budapest, Hungary); *N,N*-bis(β-chloroethyl)-*N,O*-propylenephosphodiamide (Endoxan, Asta-Werke A.G., West Germany). Nicotinamide-adenine dinucleotide (NAD) in the reduced and oxidized form (C.F. Boehringer & Soehne, Mannheim, Germany). Pyridoxal phosphate (Fluka, Buchs, Switzerland). 2-Oxoglutaric acid (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). L-Aspartic acid (B.D.H., Poole, England). Dowex 50 X4 (H^+ form) 200 - 400 mesh (Fluka, Switzerland). A sample of 7-ethylguanine was a gift from Dr. P. Brookes (Chester Beatty Research Institute, London).

Animals. Wistar albino male rats of 150 - 300 g. body weight and albino male mice R_3 weighing 25 - 30 g. inoculated with the Ehrlich ascites 4 were used.

The rats were given mannitol nitrogen mustard (1,6-bis(β-chloroethylamine)--1,6-deoxy-D-mannitol dihydrochloride) intraperitoneally in various total doses: 1.5 mg./kg. body wt. in a single dose, 6 mg./kg. body wt. in six daily doses, or 160 mg./kg. body wt. in two daily doses. The propylenephosphodiamide derivative of nitrogen mustard (*N,N*-bis(β-chloroethyl)-*N,O*-propylenephosphodiamide) was given to rats in an amount of 240 mg./kg. body wt. in four daily doses. The rats were killed 24 hr. after the last injection. The mice, 48 hr. after the inoculation with Ehrlich ascites cells, were injected with 0.9 mg./kg. body wt. of nitrogen mustard HN2 (methyl bis(β-chloroethyl)amine hydrochloride), in five daily doses.

Enzymic preparations. Ehrlich ascites cells were collected on the seventh day after inoculation from both the control and HN2-treated mice. The cells were washed three times according to Wu & Racker [19] with 4 volumes of a wash medium composed of 0.01 M-tris, pH 7.4, 4 mM-potassium phosphate, pH 7.4, and 0.14 M-NaCl. The contaminating erythrocytes were removed after Green & Bodansky [8] by suspending the sediment in 5 vol. of ice-cold 0.04 M-NaCl. The washed cells were suspended in 4 vol. of 0.05 M-phosphate buffer, pH 7.6, then frozen and disrupted after thawing in a tight glass homogenizer with silica oxide, or disintegrated in an M.S.E. ultrasonic disintegrator in the cold (60 watts, 20 kc, 5 min.). Mitochondria from rat liver and Ehrlich ascites cells were prepared in 0.25 M-sucrose after Hogeboom [10] and homogenized in 9 vol. of 0.05 M-phosphate buffer, pH 7.6. Several cycles of freezing and thawing were applied to disrupt mitochondrial membranes. This procedure was of special importance for the determination of reductive amination of glutamate dehydrogenase activity because of marked impermeability of mitochondrial membranes to reduced NAD [15]. As in rat liver mitochondria the enzymic activity decreased markedly after a few days, the mitochondria were used for the measurement within three days after preparation.

Enzyme assays. The glutamate dehydrogenase activity was assayed by the 2-oxoglutarate-dependent oxidation of reduced NAD in the presence of ammonia. The reaction mixture contained in 3 ml.: 0.5 μmole of reduced NAD, 20 μmoles of 2-oxoglutarate, 90 μmoles of NH_4Cl, and enzymic protein (70 - 90 μg.) in 0.2 M-phosphate buffer, pH 7.6. In rat liver mitochondria the activity was determined by measuring the oxidation of reduced NAD at 340 mμ at 1-min. intervals at 37°. In the homogenates and mitochondria of ascites cells the activity was calculated from the amount of oxidized NAD formed during a 10-min. incubation period at 37.5°, NAD being measured in the form of NAD-KCN complex at 325 mμ. It was found that oxidation of reduced NAD, under the experimental conditions used, was proportional to time during 10 min. of incubation. In the controls, 2-oxoglutarate was omitted.

Determination of NAD glycohydrolase activity was based on spectrophotometric measurement of the decrease in extinction at 325 mμ of the NAD-KCN complex [4] after a 10-min. incubation of enzymic protein (0.2 - 0.4 mg.) with oxidized NAD (0.5 μmole) in 1.5 ml. of 0.05 M-phosphate buffer, pH 7.6, at 37°, followed by the addition of 1.5 ml. of 2 M-KCN. In the case of ascites cells, a 10% suspension of disrupted cells (0.2 ml.) after incubation with NAD (1 μmole in 1.5 ml.) for 10 min. at 37° was deproteinized by the addition of an equal volume of 10% trichloroacetic acid. Then 1.5 ml. of 2 M-KCN was added to the 1.5 ml. sample of the deproteinized solution.

The activity of aspartate - 2-oxoglutarate transaminase was determined by the spectrophotometric method of Cammarata & Cohen [3] at 280 mμ. The enzymic protein (0.3 - 2.0 mg.) preincubated with 50 μg. of pyridoxal phosphate, was incubated with 20 μmoles of aspartate and 20 μmoles of 2-oxoglutarate in 0.05 M-phosphate buffer, pH 7.6, at 37°, and extinction at 280 mμ was read at 1-min. intervals for 5 min.

All the spectrophotometric determinations were performed with the Unicam S.P. 550 or Beckman CF4 spectrophotometers provided with a constant temperature cell housing.

Determination of pyridine nucleotide coenzymes. Concentration of oxidized and reduced NAD and NADP in the homogenates of rat liver and Ehrlich ascites cells was determined by the ethyl methyl ketone method of Kaplan [4] at 460 mμ in the spectrofluorimeter Optica CF_4 provided with the Zeiss high-vacuum mercury lamp HQE 4D and Optica filters A_{54} and F_1, transmitting waves in the range below 400 mμ. For determinations, fresh liver (0.5 g. portions) was immediately homogenized in 9 vol. of 5% trichloroacetic acid; Ehrlich ascites cells (1 ml. portions of 5% suspension) were homogenized in 1 vol. of 10% trichloroacetic acid. Then 0.5 ml. samples of the deproteinized extracts were used for determination of coenzymes; no extraction of trichloroacetic acid with ether was necessary. The extracts did not contain any fluorescent compounds when, prior to determinations, the coenzymes were destroyed by heating at 100° for 15 min. at pH 11.5 [11].

Analysis of purine components of RNA. Liver RNA of rats treated with the mannitol and propylenephosphodiamide derivatives of nitrogen mustard and RNA

of ascites cells of HN2-treated mice was extracted with phenol at 60° for 3 min. [16] and prepared after Kirby [12]. The isolated RNA was hydrolysed in 1 N-HCl at 100° for 1 hr. to yield purine bases and pyrimidine nucleotides [17]. The ion-exchange chromatography of the acid hydrolysate was carried out on Dowex 50 X4 (H^+ form) using 1 N-HCl or a gradient of 1 - 2.5 N-HCl for elution [5, 14]. Identification of purines was based on the absorption and fluorescence spectra and ascending paper chromatography in methanol - conc. HCl - water (7 : 2 : 1, by vol).

RESULTS

Table 1 illustrates the results of administration of mannitol nitrogen mustard on the activities of NAD glycohydrolase, aspartate - 2-oxoglutarate aminotransferase and glutamate dehydrogenase in rat liver mitochondria. As it can be seen, no significant differences were found between the results obtained when different types of treatment were applied, although the doses of mannitol nitrogen mustard ranged from a mild therapeutic one to LD_{50}. Therefore it seemed justified to compare mean values for all treated animals with those for controls. It appeared that there were no significant differences between the two groups in the activities of NAD glycohydrolase and glutamate dehydrogenase. The differences between the treated and non-treated animals in the activity of aspartate - 2-oxoglutarate aminotransferase were significant at 0.05 level, therefore still doubtful. The enzymic activities examined in liver mitochondria were not related to the leucocyte count in rats treated with mannitol nitrogen mustard. After a dose of 1.5 mg./kg. body wt. no changes in the number of leucocytes in the blood were observed, whereas in rats surviving an LD_{50} dose the leucocyte count was decreased by 50 - 70% (from 11 000 - 16 000 to 3000 - 7000/mm^3.).

The concentration of the two pyridine nucleotides in liver homogenates was decreased (Table 2) after treatment with mannitol nitrogen mustard. The decrease was slight but statistically significant. It was not, however, associated with any changes in glutamate metabolism in mitochondria, as judged by the activities of transaminating and deaminating enzymes.

Treatment with nitrogen mustard of mice bearing Ehrlich ascites tumour increased threefold the activity of NAD glycohydrolase in the ascites cells (Table 3). This was accompanied by a 40% decrease in concentration of pyridine nucleotides and a pronounced eightfold decrease in the activity of glutamate dehydrogenase in mitochondria. The dose of HN2 used was the same as that used by Green & Bodansky [7, 8, 9], and, as it was found, it constituted approx. 1/10 of the LD_{50} dose. The activity of aspartate - 2-oxoglutarate aminotransferase remained unchanged under these conditions.

In Ehrlich ascites cells, glutamate dehydrogenase was found to be closely bound with the cellular structures since it could not be detected in the buffer extracts from living and from acetone-dried cells. Its activity was confined to mitochondria, and in the whole homogenates it could be observed only when the cells had been exposed to ultrasonic vibrations which totally disintegrate cellular structures. In iso-

Table 1

The activity of NAD glycohydrolase, glutamate dehydrogenase and aspartate - 2-oxoglutarate aminotransferase in the liver mitochondria of rats treated with mannitol nitrogen mustard

For composition of the incubation mixture and details, see Methods.

Treatment	Exp. no.	NAD glycohydrolase (μmoles/hr./mg. protein)	Aspartate-2-oxoglutarate aminotransferase (μmoles/min./mg. protein)	Glutamate dehydrogenase (μmoles/min./mg. protein)
Control, non treated	1	0.18	0.42	0.30
	2	0.36	1.10	0.23
	3	0.96	0.83	0.27
	4	0.54	1.09	0.29
	5	0.58	0.55	0.16
	6	0.90	0.53	0.30
	7	0.45	0.55	0.15
	8	0.30	0.90	0.25
	mean	0.53	0.75	0.24
1.5 mg/kg. body wt., single dose	1	0.18	0.45	0.12
	2	0.30	0.26	0.12
	3	1.00	0.42	0.20
6 mg./kg. body wt., in 6 days per 1 mg.	1	0.66	0.66	0.26
	2	0.72	0.64	0.30
	3	1.20	0.63	0.24
90 mg./kg. body wt., in 2 days per 30 mg. and 60 mg.	1	0.24	0.50	0.17
160 mg./kg. body wt., in 2 days per 80 mg. (LD_{50} dose)	1	0.60	0.34	0.34
	2	0.36	0.40	0.22
	mean	0.58	0.48	0.22
Difference: treated *minus* non treated		—0.05	0.27	—0.02
Standard error of the difference		0.155	0.104	0.033
t value		0.33	2.58*	0.76

* Significant at 0.05 level.

lated mitochondria, the usually applied freezing and thawing technique followed by homogenization in hypotonic buffer was sufficient to disrupt their structure.

Examination of enzymic activities in the animals treated with nitrogen mustards was supplemented by an analysis of purine RNA components, which are the major sites of alkylation in nucleic acids. For this purpose, RNA acid hydrolysates were fractionated on Dowex 50 X4 (H^+) column. The elution patterns were the same in the case of RNA from liver of rats treated with mannitol or propylenediphosphate derivative of nitrogen mustard, and RNA from Ehrlich ascites cells from HN2-trea-

ted mice (Fig. 1). A peak between guanine and adenine appeared which was not observed in the hydrolysates of control RNA from both kinds of experimental material. The location of the peak corresponded to that of the methyl or ethyl deri-

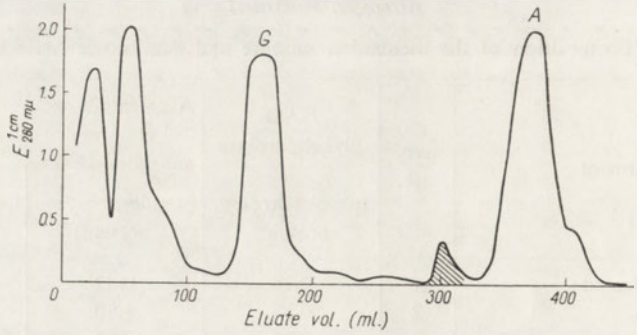

Fig. 1. Ion-exchange chromatography of acid hydrolysate of RNA from liver of rats treated with *N,N*-bis(β-chloroethyl)-*N,O*-propylenephosphodiamide in LD_{50} dose. A Dowex 50 (H^+ form) column (1 cm. × 10 cm.) with gradient elution from 1 N to 2.5 N-HCl was used. For experimental details see text. (*G*), Guanine; (*A*), adenine. The hatched area corresponds to the peak appearing after treatment.

vatives of guanine obtained upon ion-exchange chromatography of acid hydrolysate of RNA from rats treated with methyl, ethyl or butyl nitrosamines [6, 15]. However, the fraction studied did not show the absorption maxima characteristic for 7-ethylguanine (249 and 274 mμ at pH 2.0) which is usually obtained upon

Table 2

The effect of mannitol nitrogen mustard on concentration of pyridine nucleotides in rat liver homogenate

The rats were treated with mannitol nitrogen mustard in two daily doses of 160 mg./kg. body wt. The pyridine nucleotides were measured fluorimetrically according to Ciotti & Kaplan [4] and expressed in μg./g. of liver. The values are averages of five experiments, each based on 2 - 3 determinations ±S.E.

Rat	μg./g.liver	Difference		S. E. of the difference	*t*
		(μg.)	(%)		
Control	408 ± 16.4				
Treated	353 ± 10.6	—55	—13	14	3.93

treatment of RNA with alkylating agents [6, 15, 14]. It showed also higher R_F value in the methanol - conc. HCl - water system. This derivative obtained under our experimental conditions has not been identified. Nevertheless, its appearance in RNA of rat liver, in which no changes in the enzymic activities after treatment were observed, and in RNA of ascites cells, the enzymic activities of which were affected by HN2 treatment, indicate that in the animals treated with nitrogen mustards

Table 3

The activity of NAD glycohydrolase, aspartate - 2-oxoglutarate aminotransferase, glutamate dehydrogenase and concentration of pyridine nucleotides in Ehrlich ascites cells from HN2-treated and non-treated mice

For composition of incubation mixtures and details, see Methods. The values are averages, ± S.E., of four experiments, performed each with cells collected from 2 - 5 mice and based on 2 - 3 determinations. The activities of NAD glycohydrolase and aminotransferase refer to homogenates and the activity of glutamate dehydrogenase to mitochondria of ascites cells. The concentration of pyridine nucleotides was determined in cells spun down at 4500 rev./min. for 5 min.

Ascites cells from mice	NAD glycohydrolase (μmoles/hr./mg. N)	Aspartate - 2--oxoglutarate aminotransferase (μmoles/min./mg. N)	Glutamate dehydrogenase (μmoles/min./mg. N)	Concn. of pyridine nucleotides (μg./g. cells)
Control	0.60 ± 0.08	0.45 ± 0.07	0.16 ± 0.01	160
HN2-treated	2.13 ± 0.15	0.40 ± 0.05	0.02 ± 0.003	100

the changes occurring in RNA are independent from changes in the activity of the enzymes studied.

DISCUSSION

Treatment of rats with an LD_{50} dose of mannitol nitrogen mustard was without effect on the activities of NAD glycohydrolase, glutamate dehydrogenase and aspartate - 2-oxoglutarate aminotransferase in liver mitochondria, although this treatment caused a 50 - 70% decrease of the leucocyte count in the blood. This seems to suggest that nitrogen mustards affect various organs in a different way and that they may act specifically on the endoreticular system, in which blood cells are produced.

When the experiments on liver mitochondria of rats treated with mannitol nitrogen mustard were completed, a paper by Green & Bodansky [19] appeared which proved univocally the selective action of nitrogen mustards in mice bearing the Ehrlich ascites tumour. The increase of NAD glycohydrolase activity and the resulting decrease in concentration of pyridine nucleotides after HN2 treatment were found only in the ascites cells. No such changes were observed in the liver, kidney and erythrocytes of HN2-treated mice bearing Ehrlich ascites tumour.

Thus Ehrlich ascites cells constitute a very specific and interesting model for studying the role of NAD glycohydrolase in cellular metabolism by controlling the concentration of NAD. Green & Bodansky found that a threefold decrease in concentration of pyridine nucleotides in ascites cells due to the enhanced activity of NAD glycohydrolase, was not reflected in the activities of NAD-dependent lactate dehydrogenase, α-glycerophosphate dehydrogenase and glyceraldehydephosphate dehydrogenase. However, the actual rate of lactate formation was markedly reduced and it was proportional to the decrease in concentration of pyridine nucleotides [7].

A distinct decrease observed by us in the activity of glutamate dehydrogenase in Ehrlich ascites cells upon HN2 treatment is an exception among the enzymes studied so far. This decrease could be due to the direct inhibiting action of HN2 on this particular enzymic protein. It may be, however, that the lowered concentration of coenzyme is responsible for the decrease of this activity. Since the activity of aspartate - 2-oxoglutarate aminotransferase was unaffected by HN2 treatment, further studies are needed to establish whether the overall metabolism is compensated *via* the transamination route.

Special attention should be drawn to the fact that changes observed in RNA upon treatment with nitrogen mustards seem to be not related to the enzymic activities studied.

REFERENCES

[1] Amborst V. & Maas H., - *Naturwissenchaften* **51**, 271, 1964.
[2] Brookes P. & Lawley P. D. - *J. Chem. Soc.* **766**, 3923, 1951.
[3] Cammarata P. S. & Cohen P. P. - *J. Biol. Chem.* **176**, 715, 1948.
[4] Ciotti M. M. & Kaplan N. O., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 890 and 896. Academic Press, New York 1955.
[5] Craddock V. M. & Magee P. N. - *Biochem. J.* **89**, 32, 1963.
[6] Fraser J. M. - *Fed. Proc.* **19**, 3595, 1960.
[7] Green S. & Bodansky O. - *J. Biol. Chem.* **237**, 1752, 1962.
[8] Green S. & Bodansky O. - *J. Biol. Chem.* **238**, 2119, 1963.
[9] Green S. & Bodansky O. - *J. Biol. Chem.* **239**, 2613, 1964.
[10] Hogeboom P. H., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 1, p. 16. Academic Press, New York 1950.
[11] Kaplan N. O., Colowick S. P. & Barnes C. C. - *J. Biol. Chem.* **191**, 461, 1951.
[12] Kirby K. S. - *Biochem. J.* **64**, 405, 1956.
[13] Lawley P.D. & Brookes P. - *Biochem. J.* **89**, 127, 1963.
[14] Magee P. N. & Lee K. Y. - *Biochem. J.* **91**, 35, 1964.
[15] Purvis J. L. & Lövenstein J. M. - *J. Biol. Chem.* **236**, 2794, 1961.
[16] Sherrer K. & Darnell J. E. - *Biochem. Biophys. Res. Commun.* **7**, 486, 1962.
[17] Visher E. & Chargaff E. - *J. Biol. Chem.* **176**, 715, 1948.
[18] Wheeler G. P. - *Cancer Res.* **22**, 651, 1962.
[19] Wu R. & Racker E. - *J. Biol. Chem.* **234**, 1029, 1959.

WPŁYW IPERYTU AZOTOWEGO NA METABOLIZM GLUTAMINIANU W MITOCHONDRIACH WĄTROBY SZCZURA I KOMÓRKACH NOWOTWOROWYCH EHRLICHA

Streszczenie

1. Mannitolowa pochodna iperytu azotowego podana szczurom w dawkach LD_{50} nie wywołuje zmian w aktywności NAD-glikohydrolazy, dehydrogenazy glutaminianu i aminotransferazy asparaginian : α-ketoglutaran w mitochondriach wątroby, chociaż ilość białych ciałek krwi jest zmniejszona o 50 - 70%.

2. W komórkach nowotworowych Ehrlicha u myszy, którym podawano iperyt azotowy, wykazano wzrost czynności NAD-glikohydrolazy oraz 8-krotny spadek czynności dehydrogenazy glutaminianu. Aktywność aminotransferazy asparaginian : α-ketoglutaran pozostała bez zmian. Stwierdzono zmniejszenie się ilości nukleotydów pirydynowych.

3. Otrzymane wyniki zestawiono ze zmianami obserwowanymi w składzie zasad purynowych w RNA.

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W. OSTROWSKI and A. KRAWCZYK

HIGH-VOLTAGE PAPER ELECTROPHORESIS. APPARATUS AND APPLICATION TO TWO-DIMENSIONAL SEPARATIONS

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1. Construction of a cooled chamber for high-voltage paper electrophoresis with potential gradient of up to 100 v/cm. has been described. The apparatus can be used for analytic or preparative separation, and for two-dimensional separation, ensuring constant temperature and moisture of filter paper. 2. Examples of two-dimensional separation of the peptides of egg yolk flavoprotein and human and horse oxyhaemoglobins are presented.

High-voltage paper electrophoresis is characterized by high resolving power for low molecular weight compounds and can be used for quantitative assays of very small amounts of substances, as well as for preparative purposes. Separation is achieved after relatively short periods of time and better resolution can be obtained since diffusion and electroosmotic flow are limited by the high-potential gradient applied. At higher potentials, the velocity of ion migration increases linearly, while Joule's heat increases with the square of field intensity; therefore an efficient cooling system is of primary importance. The use of high-voltage paper electrophoresis technique requires particularly rapid removal of heat from the filter paper by conduction and not as heat of evaporation. During evaporation the solvent is distilled and condenses as drops on the walls of the apparatus. Inefficient removal of heat from the filter paper disturbs the migration of the separated zones as a result both of desiccation of the paper and falling of drops of the solvent on the separating surface.

Two types of apparatus for high-voltage electrophoresis which are currently in use, are provided with the following heat-removing devices: (a), the filter paper is immersed in an organic solvent immiscible with water, through which heat is removed [9, 14, 7]; (b), the filter paper is placed horizontally on a glass plate or insulated metal plate and is cooled from one [15, 16, 13] or from both sides [10, 1, 5]. However, in this case technical difficulties in the construction of the apparatus are encountered.

A very simple model of high-voltage electrophoresis apparatus has been elaborated in this laboratory; it permits to apply potential gradients of up to 100 v/cm., is provided with an efficient cooling system and gives reproducible results of separation. Original sheet of Whatman filter paper can be inserted in the electrophoretic

chamber and practically its whole surface is utilized for the separation. The apparatus, which has been used successfully for some years for fractionation of amino acids, peptides and nucleotides, is described in this paper.

EXPERIMENTAL

Apparatus. Its construction is based on the "hanging strip" principle, but the whole sheet of filter paper is placed on a bent thin glass plate forming a wedge-like structure; the plate is cooled from within by a cooling solution continuously injected under pressure. Construction of the apparatus is shown in Fig. 1. On the basal plexiglass plate 65×45 cm. (*9*), a bent at an angle of 30° glass plate is placed (*3*), the

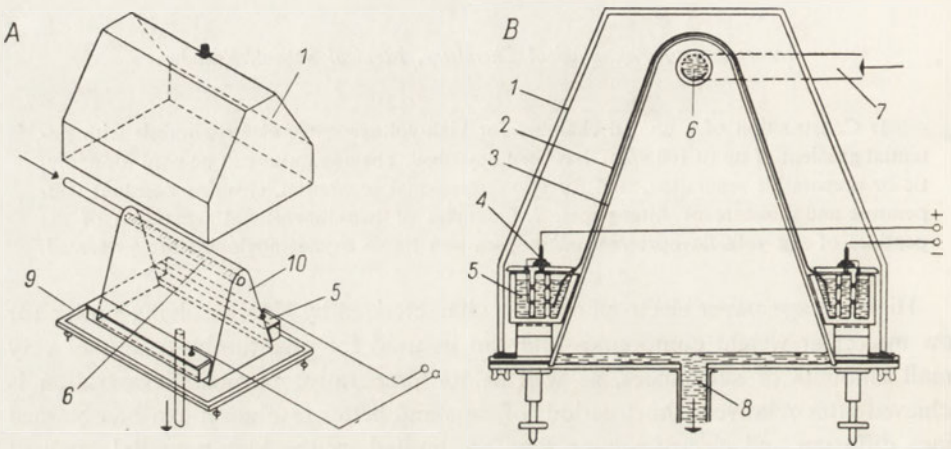

Fig. 1. Scheme of the apparatus for high-voltage paper electrophoresis; *A*, with raised cover; *B*, cross-section. (*1*), Plastic cover; (*2*), filter paper; (*3*), wedge-shaped glass plate; (4), connection with source of current; (*5*), electrode vessel; (*6*), metal tube with holes; (*7*), sprayer; (*8*), outlet for the coolant; (*9*), basal plexiglass plate; (*10*), lateral plexiglass wall.

radius of the curvature being 3.5 cm. and the height of the wedge 30 cm. The glass plate is 3 mm. thick and its surface is very even and smooth. The space under the glass plate is closed by lateral plexiglass walls (*10*) packed with rubber lists. Inside, just under the bend of the glass wedge, there is a metal tube (*6*), 30 mm. in diameter, with a row of small holes, 0.5 mm. in diameter, in its upper surface; through these holes the cooling liquid is injected under pressure to spray the inner surface of the glass plate. Through an outlet (*8*) in the basal plate the cooling liquid returns to the thermostated refrigeration unit (Fig. 2). Along both edges of the glass plate electrode vessels, 48×6×5 cm., are placed (*5*), with inwoven platinum wire electrodes. The electrodes are connected with the source of the high-voltage current through wires and clamps (*4*). The whole apparatus is enclosed in a plastic cover (*1*) which is tightly fastened to the basal plate with two screws.

The cooling liquid is composed of aqueous 50% glycerol cooled in a 60-litre coolant tank with double insulated walls, made from acid-proof tin plate. The so-

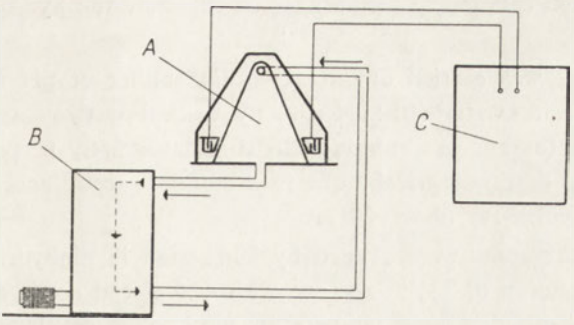

Fig. 2. Block diagram of the connections of the apparatus: *A*, electrophoretic chamber; *B*, thermostated refrigeration unit; *C*, source of high-voltage current.

lution is cooled using a compressor with a yield of about 1500 Kcal/hr., and a thermoregulator permitting to obtain temperatures ranging from +5° to -20°. The block diagram of the connections of the cooling system with the electrophoresis apparatus is shown in Fig. 2. The liquid from the reservoir is pumped into the sprayer (*7*) under pressure of about 1 atm.

The potential gradient for electrophoresis is provided using a stabilizer and electronic rectifier which gives output up to 100 mA at 100 v/cm. Raising of the cover (*1*), automatically interrupts the primary circuit of the power unit.

Preparation of peptides. Peptides were obtained by enzymic digestion of pure preparations of riboflavin flavoprotein from egg yolk [17] and human and horse oxyhaemoglobins prepared by the method of Ingram [6]. The proteins were dissolved in water, pH of the solution was adjusted with ammonia to pH 8.2 and crystalline trypsin or subtilysin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was added in the proportion of 1 : 50 by weight (enzyme : substrate). The mixture was incubated at 30° for 24 hr., 0.05 M-ammonia solution being added from time to time to maintain the pH value at about 8. After incubation, the solution was adjusted with acetic acid to pH 4.0, heated to precipitate the undigested protein and centrifuged. The clear supernatant was evaporated *in vacuo*, the dry residue dissolved in a small volume of water and submitted to electrophoresis.

Procedure. A sheet of Whatman no. 1 or 3 MM filter paper was placed in the apparatus in such a manner that the middle of the sheet was situated on the top of the bent glass wedge (Fig. 1*B*). Then the paper was rinsed with buffer, starting from the middle of the sheet. After a few minutes, when the paper was uniformly wet, it was pressed to the glass surface by means of a piece of filter paper using a rubber roller to remove air bubbles and excess of liquid. The filter paper applied in this manner adheres closely to the glass plate. Solution to be tested was then applied in small portions using a Hamilton microsyringe (Whittier, California). About 20 μl. of solution containing 0.05 μmole of each compound tested was applied per 1 cm. along the top line of filter paper. The paper was then connected with the electrode vessels by means of Whatman 3 MM filter paper strips, either

directly or through cellophane membranes to eliminate the hydrodynamic flow of the liquid [10].

Electrophoresis was carried out in a pyridine buffer of pH 6.5 composed of pyridine - acetic acid - water (10 : 0.4 : 90, by vol.). For two-way separation, the dried electrophoretogram was subjected to chromatography perpendicular to first separation by the descending technique in *n*-butanol - acetic acid - water (4 : 1 : 5 or 3 : 1 : 1, by vol.), for about 20 hr.

Electrochromatograms were stained by immersion in ninhydrin (0.5% in acetone) or isatin solution (0.5% in acetone with 4% glacial acetic acid added). The paper was then air-dried at room temperature until colour developed. After staining with ninhydrin, the chromatograms were fixed by immersion in 1% solution of copper nitrate in acetone.

RESULTS AND DISCUSSION

Separation of a mixture of amino acids possessing different isoelectric points is presented in Fig. 3. The electrophoresis was carried out for 30 - 150 min. at a potential gradient of 30 - 70 v/cm., at 50 - 80 mA, and temperature of the

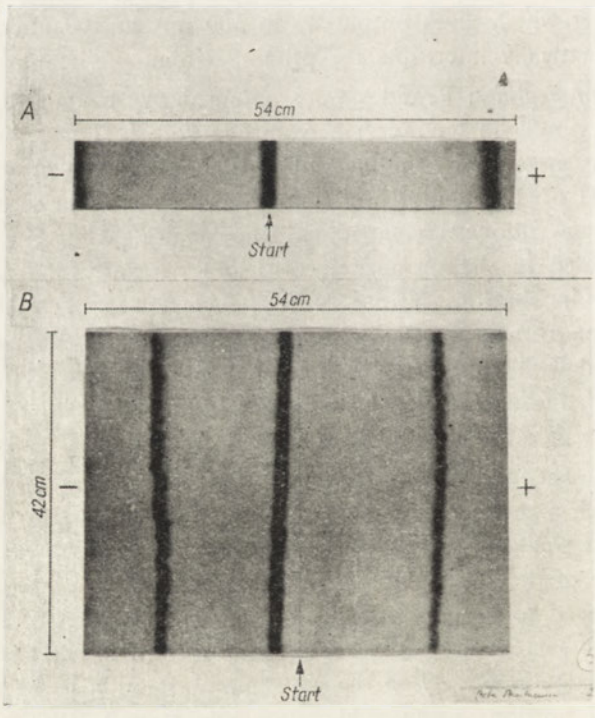

Fig. 3. Separation by high-voltage paper electrophoresis of a mixture of glutamic acid, alanine and histidine, on Whatman no. 1 filter paper in pyridine buffer, pH 6.5; 3000 v, 50 mA, 150 min., -1°. *A*, The influence of the duration time of the electrophoresis upon the shape of zones. *B*, The influence of the manner of application of the sample.

cooling liquid 0° to -5°. As can be seen, the electrophoretic zones were not distorted even when their length exceeded 40 cm. and the migration distance was about 25 cm. This indicates that the glass plate of the apparatus was evenly cooled, the filter paper was not frozen to the glass and that evaporation was not too rapid. Cremer & Tiselius [4] demonstrated that temperature differences of 1° between different points on the filter paper cause about 3% differences in electrophoretic mobility. The yield of cooling depends mainly on the thickness of the plate; the thinner the plate, the higher the temperature of the cooling solution that can be used. Adhesion of the moist filter paper to the surface of the plate is ensured by its own weight. Provided the plate has a sufficiently even and smooth surface, contact between the filter paper and glass is close, making possible rapid exchange of heat.

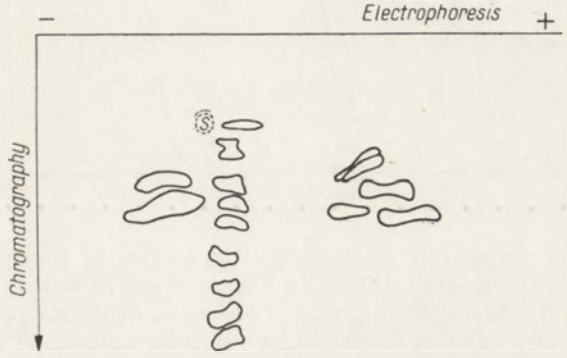

Fig. 4. Map of flavoprotein tryptic peptides. Electrophoresis: pyridine buffer, pH 6.5, 2100 v, 30 mA, 150 min. Chromatography: *n*-butanol - acetic acid - water (4 : 1 : 5, by vol.), Whatman no. 1 filter paper, ninhydrin staining. *S*, Point of application of the sample.

Depending on the buffer used, its ionic strength, and type of filter paper, such a temperature of the cooling liquid should be chosen that the temperature of the moist filter paper will be from 2 to 4°.

Further, riboflavin flavoprotein digestion products were submitted to two-dimensional separation. The isolation and some physico-chemical properties of riboflavin flavoprotein from egg yolk were reported previously [12, 17], and in the present work an attempt was made to determine the structure of this protein and the manner of riboflavin binding. The purified preparation was submitted to enzymic hydrolysis and the peptides obtained were separated by two-dimensional technique (Fig. 4). After trypsin digestion 16 peptides were obtained but none of them was bound to flavin indicating that the digestion abolished the riboflavin-binding ability. These results were confirmed by gel-filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden), when the products obtained by trypsin or subtilysin digestion of flavoprotein were filtrated on the Sephadex column (Fig. 5). As can be seen free riboflavin migrated as a separate fraction devoid of peptides, and the vitamin B_2--peptide complex was not observed in any of the obtained fractions.

Peptides obtained by trypsin hydrolysis of human and horse haemoglobins after two-dimensional separation and staining with ninhydrin exhibited no essential differences in the distribution of the spots on the electrochromatogram. The number and distribution of peptides were similar, as the number of lysine and arginine residues in both haemoglobins is the same [2]. However, when isatin staining was

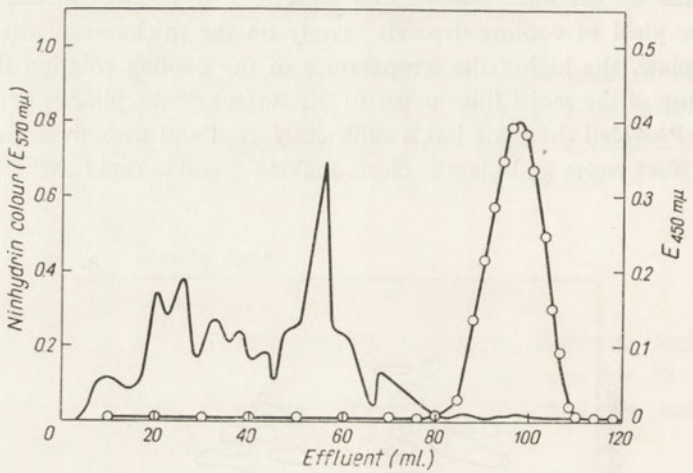

Fig. 5. Separation of flavoprotein subtilysin-digestion products by gel-filtration on Sephadex G-25 column (1.5×120 cm.). Sample (10 mg. in 2 ml.) dissolved in water and eluted with water at room temperature. In the eluates, (—), peptides were tested by the ninhydrin reagent [11] and (○) free riboflavin was determined by measuring extinction at 450 mμ.

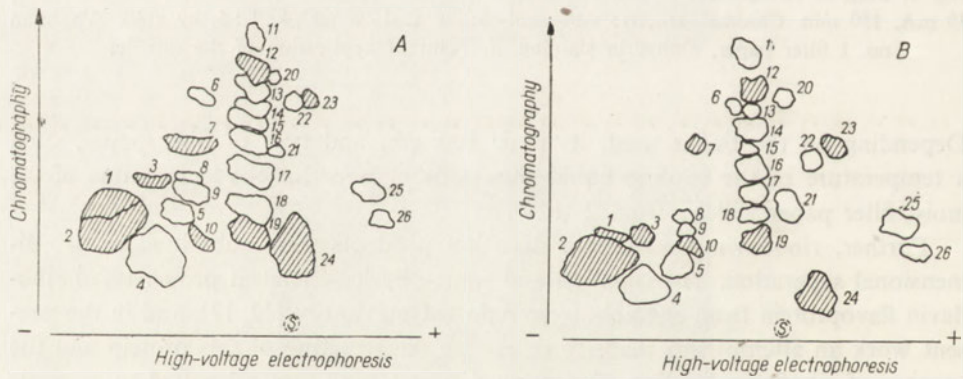

Fig. 6. Two-dimensional separation of peptides obtained by trypsin digestion of (*A*), human HbO_2 and (*B*), horse HbO_2. The corresponding peptides of the two haemoglobins which differed in colour on isatin staining, are marked by hatching. Whatman 3 MM paper. Electrophoresis: pyridine buffer, pH 6.4, 35 v/cm., 50 mA, 180 min., -3°. Chromatography: *n*-butanol-acetic acid-water (3 : 1 : 1, by vol.). *S*, Point of application of the sample.

used [8] the individual peptides differed in colour, which is known to be dependent on amino acid composition. In Fig. 6 the corresponding peptides of the two haemoglobins which differed in colour on isatin staining, are marked by hatching.

For instance, peptide no. *1* of human haemoglobin was stained blue, and nos. *10*, *12*, *19* and *24*, pink. The analogous peptides of horse haemoglobin were stained pink, blue, dark-violet, violet-pink and violet-pink, respectively. This indicates that some of the peptides of both haemoglobins, despite similar electric charges and similar molecular weights, differ in amino acid composition. This is in agreement with the analytic results of Braunitzer & Matsuda [3] who found that about 10% of the amino acid residues in the α chain of horse haemoglobin occupy different positions than in the analogous chain of human haemoglobin.

The presented results indicate that two-dimensional separation of peptide mixtures by the described procedure, together with isatin staining of electrochromatograms, can greatly simplify the comparison of the primary structure of two closely similar proteins.

REFERENCES

[1] Atfield G. N. & Morris C. J. O. R. - *Biochem. J.* **81**, 606, 1961.
[2] Braunitzer G., Hilschmann N., Rudolff V., Hilse K., Liebold B. & Müller R. - *Nature* **190**, 480, 1961.
[3] Braunitzer G. & Matsuda G. - *Z. physiol. Chem.* **325**, 91, 1961.
[4] Cremer H. D. & Tiselius A. - *Biochem. Z.* **320**, 273, 1950.
[5] Gross D. - *J. Chromatogr.* **5**, 194, 1961.
[6] Ingram V. M. - *Biochim. Biophys. Acta* **28**, 539, 1958.
[7] Katz A. M., Dreyer J. & Anfinsen Ch. B. - *J. Biol. Chem.* **234**, 2897, 1955.
[8] Krawczyk A. - *Bull. Acad. Polon. Sci., Ser. Biol.* **10**, 87, 1962.
[9] Michl H. - *Mhefte Chem.* **82**, 489, 1951.
[10] Michl H. - *Mhefte Chem.* **83**, 737, 1952.
[11] Moore S. & Stein W. H. - *J. Biol. Chem.* **211**, 907, 1954.
[12] Ostrowski W. & Krawczyk A. - *Acta Chem. Scand.* **17**, Suppl. 241, 1963.
[13] Prusik Z. & Keil B. - *Collection Czechoslov. Chem. Commun.* **25**, 2049, 1960.
[14] Turba F., Pelzer H. & Schuster H. - *Z. physiol. Chem.* **296**, 97, 1954.
[15] Werner G. & Westphal O. - *Angew. Chem.* **67**, 251, 1955.
[16] Wieland Th. & Pfleiderer G. - *Angew. Chem.* **67**, 257, 1955.
[17] Żak Z. & Ostrowski W. - *Acta Biochim. Polon.* **10**, 427, 1963.

ELEKTROFOREZA WYSOKONAPIĘCIOWA NA BIBULE APARATURA I JEJ ZASTOSOWANIE DO ROZDZIAŁU DWUKIERUNKOWEGO

Streszczenie

1. Opisano konstrukcję prostej komory chłodzonej do wysokonapięciowej elektroforezy bibułowej, w której można stosować spadki potencjału do 100 V/cm. Aparat może być zastosowany do rozdziału analitycznego i preparatywnego na większą skalę oraz do rozdziału dwukierunkowego przy zapewnieniu stałej temperatury i wilgotności bibuły.

2. Podano przykłady rozdziału dwukierunkowego peptydów flawoproteidu żółtka jaja oraz oksyhemoglobiny ludzkiej i końskiej.

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OLGA SZYMONA and T. SZUMIŁO

ADENOSINE TRIPHOSPHATE AND INORGANIC POLYPHOSPHATE FRUCTOKINASES OF *MYCOBACTERIUM PHLEI*

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1. Cell-free extracts from fructose-grown *M. phlei* were found to be capable of phosphorylating fructose at C-6 with either ATP or inorganic polyphosphate (Graham's salt). The fructokinases have been purified 15-fold and separated from specific glucokinases by Sephadex G-100 gel filtration. 2. The enzyme preparation required Mg^{2+} and was most active at pH 8.5 with KCl added. It was heat-labile and underwent total denaturation upon 1-min. exposure to 70°. 3. Experimental data suggest that inorganic polyphosphate is utilized by an enzyme different from the fructokinase utilizing ATP. 4. In contrast to mycobacterial hexokinases, a phosphofructokinase present in a preparation from *M. phlei* proved to be active with ATP but not with polyphosphate.

Mycobacteria can utilize for growth a variety of sugars, little, however, is known about the phosphotransferases involved in these processes with the exception of enzymes phosphorylating glucose and glucosamine [20]. In the present work the phosphorylation of fructose was studied to determine whether *M. phlei* possesses, in addition to the two specific glucokinases described earlier [20, 21], a fructokinase capable of utilizing inorganic polyphosphate. The presence of fructokinase in bacteria was studied by Doudoroff *et al.* [6], Palleroni *et al.* [17] and Domagk & Horecker [5].

Part of this study has been presented at the Third Symposium of the Polish Biochemical Society in Kazimierz, September 1964.

EXPERIMENTAL

Micro-organism. The culture of *Mycobacterium phlei* has been maintained for several years by mass transfers on solid Lowenstein media. For experiments *M. phlei* was grown at 37° on a synthetic medium [9] containing 4% fructose, 1% glutamic acid, 0.2% citric acid, 0.05% $MgSO_4 \cdot 7H_20$, 0.05% KH_2PO_4, 0.005% ferric ammonium citrate and 0.002% $ZnSO_4 \cdot 7H_2O$. The pH was adjusted to 7.4 with 1 N-KOH before sterilization. The medium was dispensed in large Fernbach flasks. Sterile fructose (20%) was added aseptically to a final concentration of 4%. In several cases glucose was substituted for fructose. Usually, one 330-ml.

portion of the medium was inoculated with a suspension of 7-day-old bacteria collected from one Lowenstein slant culture. After about 10 days of static growth, the cells forming a pellicle on the surface were harvested by centrifuging or filtration through gauze, washed twice with water and used immediately for experiments or stored in the frozen state until needed.

Chemicals. Inorganic polyphosphate (poly-P) was a synthetic product (Graham's salt) kindly supplied by Prof. Dr. J. P. Ebel. The substance was dissolved in water, some insoluble material removed by centrifuging and low-molecular impurities dialysed off against water. The resulting solution was neutralized with NaOH and diluted to contain 10 μmoles of easily hydrolysable phosphate per 0.1 ml. When tested with purified poly-P glucokinase, the rate of glucose phosphorylation was identical with that previously observed with Thilo's poly-P preparation [20].

ATP (sodium salt) was a product of the Sigma Chemical Company (St. Louis, U.S.A.). D-Fructose was purchased from B.D.H. (Poole, England) and crystalline D-fructose from Merck (Darmstadt, Germany). Tris(hydroxymethyl)-aminomethane was obtained from L. Light Co. (Colnbrook, England). Deoxyribonuclease (EC 3.1.4.5) was obtained from the Mann Research Lab. (New York, U.S.A.) and ribonuclease (EC 2.7.7.16) from B.D.H. (Poole, England). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was prepared from yeast by the method of Kornberg & Horecker [13]. Aldolase (EC 4.1.2.7), phosphoglyceraldehyde dehydrogenase (EC 1.2.1.9) and nicotinamide nucleotides (NAD and NADP) were products of Boehringer & Soehne (Mannheim, Germany). Sephadex G-25 and G-100 (medium) were purchased from Pharmacia, Uppsala. Whatman DEAE-cellulose powder DE50 (W. & R. Balston Ltd., England) was washed twice with 0.5 N-NaOH and then with water until neutral. Calcium phosphate gel was prepared by the method of Keilin & Hartree [12]. Alumina C-γ was prepared according to Dawson & Magee [3]. For disintegration of cells levigated alumina (alundum, Norton Co., Worcester, U.S.A.) or more frequently (electro)corund no. 3/320 (A) (Biuro Sprzedaży Artykułów Ścierych, Bielsko-Biała, Poland) was employed as abrasive material. The corund was washed with 2 N-HCl and water before use. Fructose-6-phosphate and glucose-6-phosphate (B.D.H., Poole, England); fructose-1-phosphate (Boehringer & Soehne, Mannheim, Germany); fructose-diphosphate (L. Light Co., Colnbrook, England). The barium salts were dissolved in dilute HCl, Ba^{2+} precipitated with solid Na_2SO_4 and the supernatants neutralized with NaOH.

All other chemicals were reagent grade; glass-distilled water was used throughout.

Enzyme assays. Fructokinase activity was assayed, if not otherwise indicated, in a medium containing 10 mM-ATP, 5 mM-$MgCl_2$, 6 mM-fructose, 0.2 M-KCl, 0.075 M-tris-HCl buffer (pH 8.5), 15 mM-NaF and enzyme.

When inorganic poly-P served as substrate, the system contained 8 mM-acid-labile phosphorus of Graham's salt, 3 mM-$MgCl_2$, 6 mM-fructose, 0.2 M-KCl, 0.075 M-tris-HCl buffer (pH. 8.5), 15 mM-NaF and enzyme.

At the beginning and at the end of incubation at 37° suitable portions were diluted with water and treated successively with 5% $ZnSO_4$ and 0.3 N-$Ba(OH)_2$ to remove protein and phosphate esters [23]. After centrifuging the supernatants were used for fructose determination by the Roe resorcinol method [18]. The difference between the initial value of fructose and that after incubation, or between blank sample run without enzyme and that with complete system was the measure of phosphorylative activity. The unit was defined as that amount of enzyme which utilized 1 μmole fructose per 1 hr. under the above conditions.

The enzyme preparations were examined for the presence of phosphatase activity, the phosphorus being estimated in trichloroacetic acid-deproteinized samples according to the Fiske-Subbarow method [7] with methol as reducing agent.

Glucokinase activity was determined by the disappearance of acid-labile phosphorus as described earlier [20].

Glucosephosphate isomerase was tested by measuring the conversion of glucose-6-phosphate (G-6-P) into fructose-6-phosphate (F-6-P) under conditions of the fructokinase assay, phosphate donor and fructose being omitted from the reaction mixture.

Analytical methods. The extinctions were measured in a photoelectric colorimeter of Chinese production, model no. 581.

Protein was determined after Lowry *et al.* [14] and in eluates from DEAE-cellulose in a Hilger spectrophotometer, and the amount calculated according to the formula given by Kalckar [11].

Two-dimensional paper chromatography was carried out as described previously [19]. *n*-Butanol - pyridine - water (3 : 2 : 1.5, by vol.) and methanol - formic acid (88%) - water (80 : 15 : 5, by vol.) were employed as solvents. Reducing spots were located with the $AgNO_3$ - NaOH reagent [22] and phosphorus compounds after acid hydrolysis [10] by ultraviolet irradiation. Fructose phosphate esters were also detected with the resorcinol reagent [8]. Besides, glucose-6-phosphate and fructosediphosphate were identified spectrophotometrically in enzymic reactions: G-6-P with G-6-P dehydrogenase and NADP; F-1,6-P_2 with aldolase, phosphoglyceraldehyde dehydrogenase and NAD.

Preparation and activity of cell-free extracts

All the preparative procedures were performed in a cold room at 0 - 4°. Wet cells were suspended in an about equal amount by weight of 0.05 M-tris-HCl buffer (pH 7.4) and disintegrated for 20 min. in a motor-driven glass homogenizer of the Potter-Elvehjem type under cooling with ice-water. The homogenate was then centrifuged in a refrigerated MSE centrifuge for 30 min. at 10 000 rev./min. and the unbroken cells and coarse debris discarded. The supernatant fluid (cell-free extract) contained about 1.5 - 2.0 mg. protein/ml.

When such an extract was incubated with fructose and ATP under standard assay conditions (see Methods) the rate of phosphorylation was lower than 1 μmole/hr./mg. protein. Sodium fluoride was necessary to inhibit phosphatase

activity. Without the fluoride 0.3 ml. of the extract incubated for 1 hr. in a mixture containing 4.5 μmole ATP, 4 μmoles $MgCl_2$, 140 μmoles KCl and tris-HCl buffer (pH 8.5) in a total volume of 1 ml., liberated 0.9 μmole ortophosphate.

Inorganic polyphosphate could also be utilized for the phosphorylation of fructose, the rate, however, was several times lower.

In an alternative procedure, wet cells were ground in a mortar with 3 parts (by weight) of alumina [15] and subsequently extracted with cold 0.05 M-tris-HCl buffer, pH 7.4 (5 ml./g. wet wt. of cells). After centrifuging for 30 min. an extract was obtained containing about 3 mg. protein/ml. It exhibited with ATP a fructokinase activity of 0.5 μmole/hr./mg. Similar results were obtained using corund as abrasive. When 0.9 mg. protein of the extract from corund-ground cells was incubated 1 hr. with 5 μmoles ATP, 3 μmoles $MgCl_2$, 3 μmoles fructose, 60 μmoles KCl, 10 μmoles NaF and buffer in a total volume of 0.75 ml., about 0.6 μmole of fructose underwent phosphorylation. Addition of reduced glutathione or EDTA (1 mM) did not increase the activity. It appeared that the amount of fructokinase in extracts obtained either with alumina or with corund was almost equal, which is at variance with the results for glucokinase activity which greatly decreased after corund disintegration [20].

Partial purification of mycobacterial fructokinases

Extraction. Ten grams of wet cells were ground with 30 g. of corund for 3 min. and the resulting paste was extracted for 10 min. with 20 ml. of tris-HCl buffer (pH 7.4) containing 10 mM-fructose and 1 mM-EDTA. The mixture was then centrifuged in a Servall centrifuge at 3° for 40 min. at 12 000 *g*. The yellow, opalescent crude extract contained 4 - 5 mg. protein/ml.

Ammonium sulphate fractionation. The crude extract was incubated with DNase (1 mg./100 ml. extract with the addition of 0.1 m-mole of $MgCl_2$) at 10° for 20 min. and centrifuged if necessary. Thereafter, powdered ammonium sulphate was added to remove inactive material at 35% saturation and to collect proteins precipitating between 35 and 75% saturation, care being taken to maintain the pH near 7 by occasional additions of dilute ammonium hydroxide. Usually 2/3 of the crude extract proteins were recovered in the latter fraction.

In several experiments, extracts from corund-ground cells were incubated with both DNase and RNase prior to fractionation with ammonium sulphate . In such cases proteins precipitating between 45 - 55% (I), 55 - 65% (II) and 65 - 75% (III) ammonium sulphate saturation were collected, dissolved in minimum water and dialysed against three changes of buffer for 3 - 4 hr. The highest specific activity with either ATP or poly-P was found in the fraction II. However, separation of the enzyme was not satisfactory as the two other fractions contained considerable activity. Phosphatase present in the ammonium sulphate fractions, unless inhibited by addition of NaF, decreased the fructokinase assay. When 0.2 ml. of either fraction II or III were incubated with ATP-fructose mixture to which 5 μmoles F-6-P was added in a volume of 0.7 ml. for 1 hr., 2.1 and 1 μmole phosphate were liberated respectively.

Attempts to remove nucleic acids by treatment with protamine sulphate proved negative as large loss of activity occurred.

Also the addition of 0.1 - 0.5 ml. of 5% streptomycin per 2 ml. of dialysed enzyme solution (about 20 mg. protein), followed by elution of the precipitate with 0.02 M-phosphate buffer, pH 6.5, did not give positive results.

Calcium phosphate gel adsorption. One ml. of pooled fractions II and III containing 13 mg. protein was diluted with 0.03 M-acetate buffer, pH 5.8, to a protein concentration of 4 mg./ml. and 0.5 ml. of a 2-month-old calcium phosphate gel suspension (8 mg. dry wt.) was added. After being stirred for a few minutes in the cold the suspension was centrifuged and the gel sediment was eluted with 1.5 ml. of 0.5 M-KCl in 0.2 M-tris-HCl buffer, pH 8.5. About 40% and 10% of enzyme activity was recovered with ATP and poly-P respectively.

DEAE-cellulose chromatography. DEAE-cellulose was prepared as described in Methods and poured into a column (29 cm. long, 0.9 cm. intern. diam.) under continuous stirring. After equilibration with 25 mM-tris-HCl buffer, pH 7.4, a dialysed 45 - 75% ammonium sulphate-saturation fraction was applied to the top of

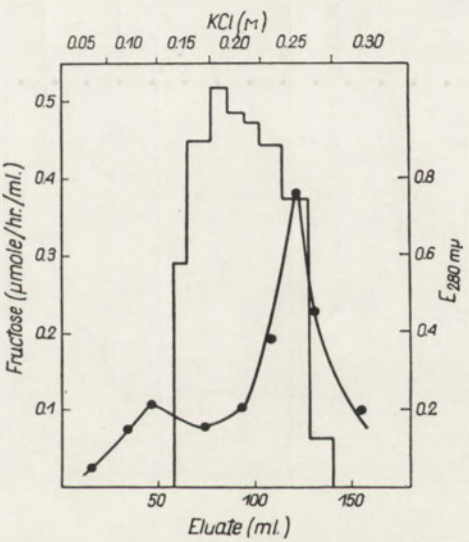

Fig. 1. Elution diagram of ATP fructokinase from DEAE-cellulose. Incubation mixtures contained: 1 ml. of the eluate, 7.5 mM-ATP, 4 mM-$MgCl_2$, 5 mM-fructose and 0.05 M-tris buffer in a total volume of 1.35 ml. KCl concentration was adjusted in each fraction to 0.25 M. (●), Extinction, representing protein concentration.

the column and washed down with 30 ml. of the starting buffer. The adsorbed proteins were then eluted with KCl in stepwise increasing concentrations. Figure 1 shows that ATP fructokinase was eluted by 0.15, 0.20 and 0.25 M-KCl, specific activity in the peak fraction reaching 4 μmoles/hr./mg. protein.

No poly-P fructokinase activity was found in the eluates. In the peak fraction, phosphatase activity was negligible and glucosephosphate isomerase was active at a very low rate of about 0.2 μmole/hr./mg. protein. When mannose was substituted for fructose, no activity could be found with ATP as shown by the Mokrasch anthrone method [16].

Sephadex gel filtration. Preliminary experiments with Sephadex G-25 showed that the hexokinases studied moved together with the bulk of proteins. Fractiona-

tion was conducted on Sephadex G-100 which had been suspended in 20 mM-tris-HCl buffer (pH 7.4) and placed in a column (39×1.1 cm.) under stirring. The dialysed 35 - 75% ammonium sulphate-saturation fraction was pipetted onto the surface of the column and the filtration procedure started at a rate of 20 - 25 ml./hr. The first protein-free effluent (12 ml.) was discarded. It was followed by 2-ml. fractions eluted with the same buffer. For determination of enzymic activities suitable samples were taken and incubated as described in Methods. Figure 2 shows the results of one of several fractionations. It is evident that fructokinases became separated from the enzymes phosphorylating glucose. In contrast to DEAE-cellulose chromatography, the use of Sephadex allowed to recover the poly-P fructoki-

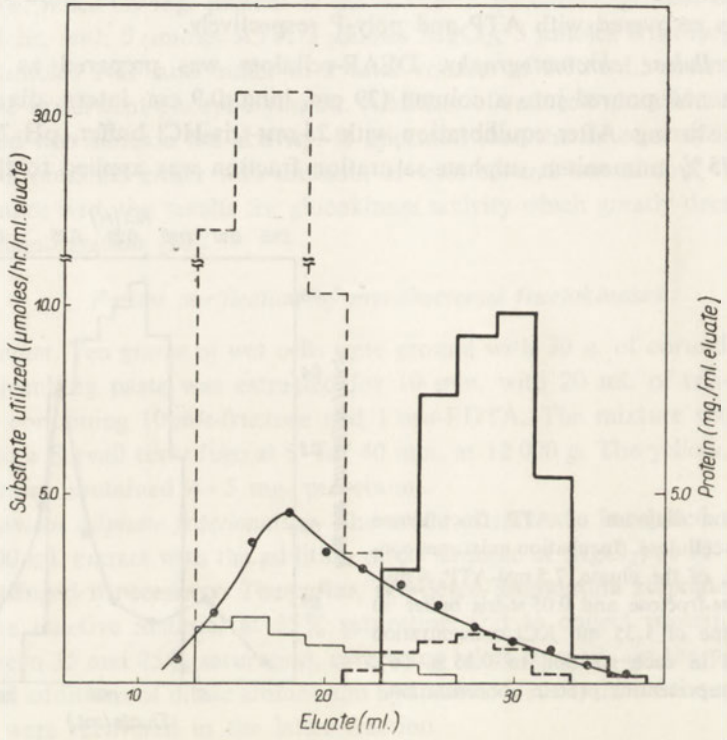

Fig. 2. Separation of mycobacterial hexokinases on Sephadex G-100 column. Dialysed 35 - 75% ammonium sulphate sat. fraction (4 ml.) containing 57 mg. protein was applied on the column (39×1.1 cm.) and treated as described in the text. (—), ATP glucokinase assay (reaction mixture contained: 0.2 ml. of the eluate, 2 mM-ATP, 4 mM-$MgCl_2$, 4 mM-glucose, 40 mM-tris buffer, pH 8.5, and 10 mM-NaF in a total volume of 0.4 ml.; incubation 1 hr. at 37°). (—), ATP fructokinase assay (reaction mixture contained: 0.25 ml. of the eluate, 4 mM-ATP, 4 mM-$MgCl_2$, 4 mM-fructose, 60 mM-KCl, 40 mM-tris buffer and 15 mM-NaF in a total volume of 0.5 ml.; incubation 30 min. at 37°). (- - -), Poly-P glucokinase assay (reaction mixture contained: 0.05 ml. of the eluate, 6 mM-poly-P, 3 mM-$MgCl_2$, 6 mM-glucose, 0.25 M-KCl and 0.075 M-tris buffer in a total volume of 0.25 ml.; incubation 30 min. at 37°). (- - -), Poly-P fructokinase assay (reaction mixture contained: 0.5 ml. of the eluate, 4 mM-poly-P, 2 mM-$MgCl_2$, 3 mM-fructose, 0.15 M-KCl and 0.03 M-tris buffer in a total volume of 0.7 ml.; incubation 1 hr. at 37°). (●), Protein concn.

nase activity. However, no separation was achieved between the enzymes utilizing poly-P and ATP, although a shift of peaks suggests that they are not identical. At the same time, a further purification was obtained; the specific activity of ATP fructokinase in the peak fraction was about 10 μmoles/hr./mg. protein, which was about 15 times more than the initial value. The activity towards poly-P was several times smaller than towards ATP.

The Sephadex eluates which exhibited fructokinase activity also contained ATPase and glucosephosphate isomerase. When 0.4 mg. protein was incubated with 4 μmoles ATP, 4 μmoles $MgCl_2$, 100 μmoles KCl and 40 μmoles tris-HCl buffer (pH 8.5) in a total volume of 0.66 ml., 0.63 μmole phosphate was liberated during 80 min. The same amount of enzyme in the presence of 2.5 μmoles G-6-P, 2 μmoles $MgCl_2$ and

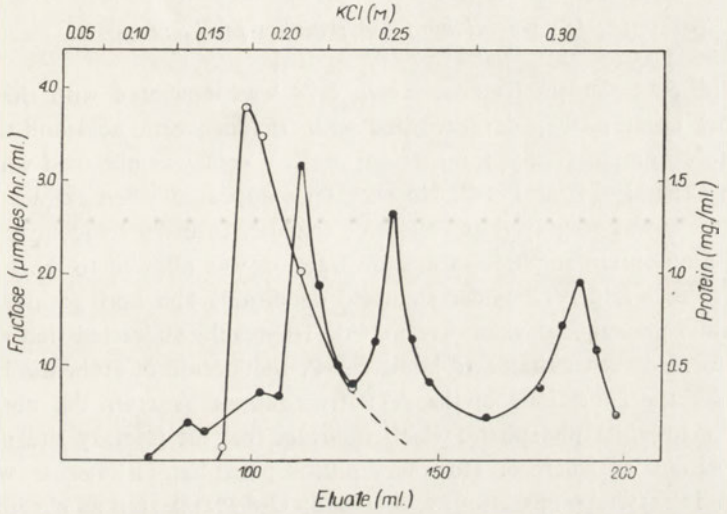

Fig. 3. Elution diagram of ATP fructokinase from DEAE-cellulose. Most active Sephadex eluates were pooled (15 ml., 70 mg. protein) and applied on the column (29×0.9 cm.); 44 mg. of protein with a total activity of 100 units were eluted by 0.15, 0.20 and 0.25 M-KCl (12 fractions, each 5 ml.). (○), Enzyme activity (reaction mixture contained: 0.5 ml. of the eluate, 3 mM-ATP, 3 mM-$MgCl_2$, 2.5 mM-fructose, 0.15 M-KCl, 0.05 M-tris buffer and 8 mM-NaF in a total volume of 0.7 ml.; incubation 30 min. at 37°). (●), Protein concentration.

60 μmoles KCl (pH 8.5) produced 0.24 μmole fructose-6-phosphate in 1 hr. as determined by the resorcinol method [18]. This result might have been influenced by some hydrolysis of hexose phosphate, since an incubation of 5 μmoles of F-6-P without NaF liberated 0.15 μmole phosphate.

In a separate experiment the Sephadex eluates with fructokinase activity were pooled and chromatographed on DEAE-cellulose. Figure 3 shows the elution diagram. ATP fructokinase appeared in the same range of KCl concentrations as in Fig. 1, while the poly-P activity was lacking.

In Table 1 the results of a typical fractionation procedure are summarized.

Table 1

Purification of ATP fructokinase from M. phlei

One unit is defined as that amount of enzyme which catalyses the phosphorylation of 1 μmole fructose/hr.

Fraction	Total units	Units/1 mg. protein
Crude extract	90	0.8
45 - 75% ammonium sulphate sat. ppt.	67	1.2
Sephadex G-100		
pooled	64	4.0
peak fraction	16	10.0

Identification of the reaction products

When the extract from fructose-grown cells was incubated with the standard ATP-fructose mixture, then deproteinized with trichloroacetic acid and the extract submitted to chromatography, a resorcinol-positive spot was obtained which corresponded to either F-6-P or F-1-P. No such spot appeared when extract from glucose-grown cells was employed as source of enzyme. In order to identify the ester, a 45 - 75% ammonium sulphate-saturation fraction was allowed to react in a mixture with fructose and ATP under standard conditions and both residual fructose and acid-labile phosphorus were determined. The results suggested that acid-stable F-6-P has formed rather than acid-labile F-1-P. Incubation of authentic F-1-P with enzyme under the conditions of the ATP fructokinase reaction did not alter the amount of acid-labile phosphate, which indicates that the primary site of transfer is the 6-position of fructose. However, glucosephosphate isomerase was found to be present in enzyme preparations and actually G-6-P appeared on chromatograms as a reducing spot non-reacting with resorcinol. Another interfering reaction could be the conversion of F-6-P into fructose diphosphate (F-1,6-P_2). According to Bastarrachea *et al.* [1] mycobacterial phosphofructokinase in crude extracts is largely inhibited but can become unmasked during purification.

To identify all the reaction products a large scale experiment was carried out with the use of ATP-fructose standard incubation mixture (pH 8.5). After 1 hr. incubation the proteins were removed with perchloric acid and the solution brought to pH 8.3 with KOH. The precipitate of $KClO_4$ was then discarded and the supernatant fractionated with barium acetate [24]. The barium-insoluble fraction was found to contain F-1,6-P_2 with some hexose monophosphate, while the barium-soluble alcohol-insoluble fraction consisted of a mixture of reducing glucose and fructose phosphate esters. In addition to the chromatographic analysis, both G-6-P and F-1,6-P_2 were further identified enzymically by reduction of nicotinamide-adenine dinucleotides in the presence of G-6-P dehydrogenase and phosphoglyceraldehyde dehydrogenase with aldolase, respectively (Fig. 4). Hexose-6-phosphate could also be found when the Sephadex eluate was incubated with fructose and

inorganic polyphosphate (Fig. 4). However, no fructose diphosphate was formed in the presence of poly-P. This result was confirmed with partially purified phosphofructokinase from *M. phlei*. The enzyme preparation was obtained by ammonium sulphate fractionation (25-40% sat.) and alumina C_γ adsorption with subsequent dialysis against 10 mM-phosphate buffer (pH 7) and 1 mM-Mg^{2+} ions [1].

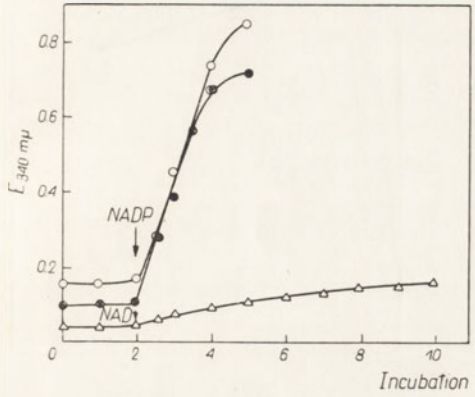

Fig. 4

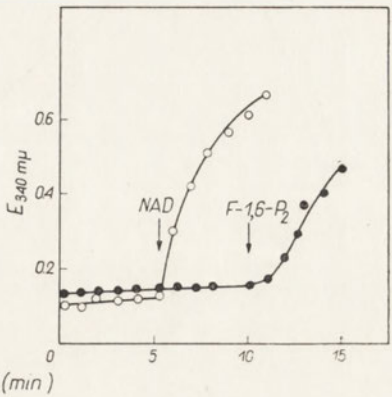

Fig. 5

Fig. 4. Identification of the products of the reaction catalysed by (○, △) ATP fructokinase and (●), poly-P fructokinase. ATP-fructokinase assay mixture was incubated with dialysed 35 - 65% $(NH_4)_2SO_4$-sat. fraction. Then the Ba-soluble alcohol-insoluble fraction was isolated and 0.5 ml. was incubated with (○), 0.07 M-tris buffer, pH 7.7, 5 mM-$MgCl_2$ and 0.1 ml. of glucose-6-phosphate dehydrogenase, 0.5 mM-NADP being added after 2 min.; total volume 2.9 ml. In the second experiment the Ba-insoluble fraction was isolated and 0.5 ml. was incubated with (△), 0.07 M-tris buffer, pH 8, 0.1 ml. of 0.2 M-cysteine, 0.2 ml. of 0.05 M-sodium arsenate, 250 μg. of phosphoglyceraldehyde dehydrogenase and 250 μg. of aldolase, 0.1 ml. of 0.01 M-NAD being added after 2 min.; total volume 2.9 ml. Poly-P fructokinase assay mixture was incubated with the Sephadex eluate (●); thereafter, the proteins were removed by addition of perchloric acid and the solution neutralized with KOH. The supernatant was then used for assay as in experiment (○).

Fig. 5. Phosphofructokinase activity in *M. phlei* with (○), ATP and (●), poly-P, as phosphate donor (for details see text). The presence of F-1,6-P_2 in the deproteinized incubation mixture was assayed enzymically using aldolase and phosphoglyceraldehyde dehydrogenase as in experiment (△) in Fig. 4. One μmole of NAD was added to each cuvette after 5 min.; 1 μmole F-1,6-P_2 was added after 10 min. to the sample incubated with poly-P.

When such an enzyme was incubated for 1 hr. with 10 mM-poly-P, 6 mM-$MgCl_2$, 5 mM-F-6-P and 0.06 M-tris-HCl buffer (pH 8), no fructose diphosphate could be detected. In a parallel sample with ATP as phosphate donor, the enzyme preparation proved to produce F-1,6-P_2 (Fig. 5).

General properties of mycobacterial fructokinases

The phosphorylation of fructose with ATP and poly-P requires both Mg^{2+} ions and KCl. Optimum concentration of KCl was found to lie in the range from 0.1 M to 0.25 M under the specified conditions (Fig. 6). At poly-P and KCl concentrations of 12 mM and 0.15 M respectively, the activity was the greatest in the presence of 2-4

mM-$MgCl_2$ (Fig. 7). At this poly-P concentration the enzyme was near saturation, since a further twofold increase in both Mg^{2+} and poly-P gave no appreciable increase of activity. However, an increase in poly-P without a corresponding increase

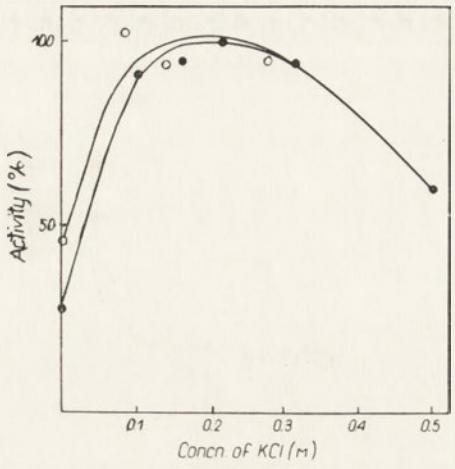

Fig. 6

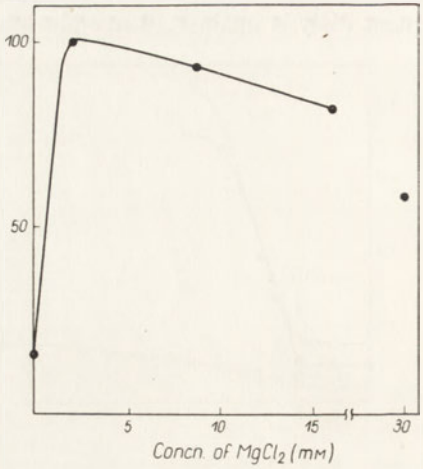

Fig. 7

Fig. 6. Effect of KCl on fructose phosphorylation with (○), ATP and (●), poly-P. Standard assay conditions except varying amounts of KCl. Enzyme: dialysed ammonium sulphate fraction II. Incubation time 30 min.

Fig. 7. Effect of $MgCl_2$ on fructose phosphorylation with poly-P. The reaction mixture contained 12 mM-poly-P, 6 mM-fructose, 0.15 M-KCl, 0.07 M-tris buffer, varying amounts of $MgCl_2$ and enzyme (ammonium sulphate fraction II, dialysed). Incubation time 1 hr.

in Mg^{2+} caused a large decrease in activity. Similarly, excess of Mg^{2+} resulted in a considerable inhibition and at 30 mM-$MgCl_2$ distinct precipitation occurred. An analogous relationship was observed between magnesium ions and ATP (Table 2).

Table 2

Effect of ATP and Mg^{2+} *on the phosphorylation of fructose*

Incubation mixtures contained dialysed ammonium sulphate fraction III, 6.7 mM-fructose, 0.1 M-KCl, 0.075 M-tris-HCl buffer (pH 8.5) and indicated amounts of ATP and Mg^{2+}.

ATP (mM)	$MgCl_2$ (mM)	Activity (%)
9.25	6	100
18.50	6	60
18.50	12	110

To test the effect of pH, a Sephadex eluate was used. A broad optimum was apparent around pH 8.5 for both fructokinases with approx. 50% and 75% activity of ATP fructokinase at pH 7.4 and 9.5, respectively. Upon addition of acetate buffer of pH 4.5, a precipitate was formed and denaturation of the enzymes occurred.

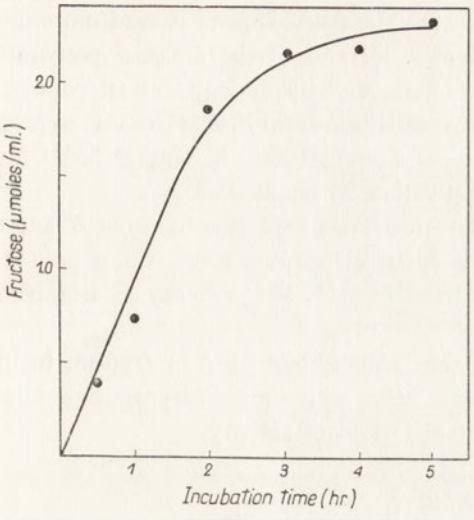

Fig. 8

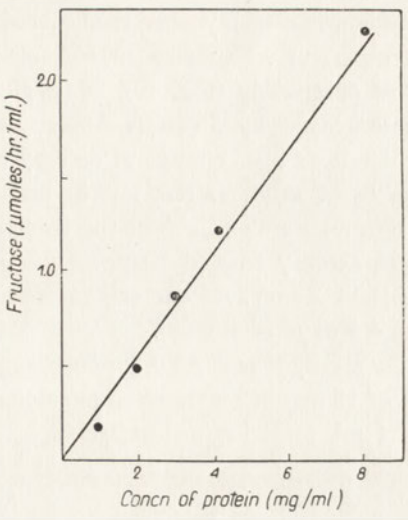

Fig. 9

Fig. 8. Time-course of fructose phosphorylation with poly-P. Standard assay conditions. Enzyme: 45 - 65% ammonium sulphate-saturation fraction, dialysed.

Fig. 9. Effect of enzyme concentration on fructose phosphorylation with poly-P. Standard assay conditions. Enzyme: 35 - 75% ammonium sulphate-saturation fraction, dialysed.

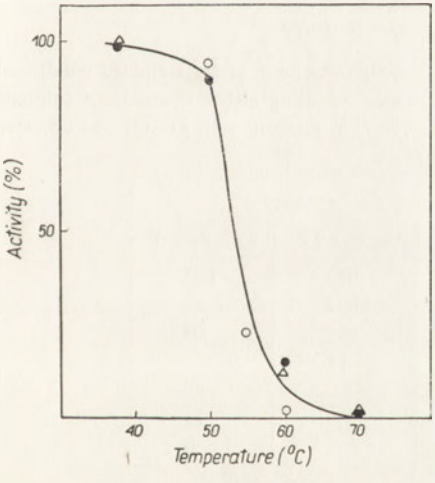

Fig. 10

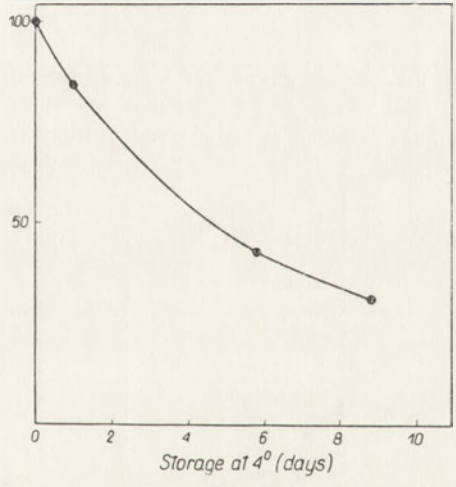

Fig. 11

Fig. 10. Heat inactivation curve of mycobacterial fructokinases. Standard assay conditions. (●), Poly-P activity (ammonium sulphate fraction I, dialysed) after 1-min. heating at indicated temperatures in the presence of 0.15 M-KCl; (Δ), in the absence of KCl. (○), ATP activity (ammonium sulphate fraction III, dialysed) after 1-min. heating in the presence of 0.15 M-KCl.

Fig. 11. Decrease in poly-P fructokinase activity during storage of Sephadex eluate at 4°. Standard assay conditions.

Figure 8 shows the time-course of fructose phosphorylation with poly-P. The reaction proceeded linearly for about 2 hr. and slowed down when 25% of poly-P was taken up. It also was proportional to enzyme concentration in the range tested (Fig. 9).

In contrast to mycobacterial glucokinases, the fructokinases proved more labile to temperature. Exposure to 60° for 1 min. inactivated the dialysed preparation almost completely (Fig. 10), while glucokinase activity under identical conditions was destroyed by 5 - 10%. Undialysed preparations of fructokinases were less heat-labile as little change of activity occurred upon 1-min. heating at 55°. On the other hand, crude extract lost its activity within 24 hr. at 0 - 2°.

In a separate experiment the Sephadex eluate was kept in solution at 4° and the kinase activity toward fructose was measured at various times using poly-P as phosphate donor. As one can calculate from Fig. 11, 50% of enzyme disappeared after 4.5 days of storage.

Both poly-P and ATP fructokinases were rapidly destroyed by trypsin. Incubation of 15 mg. of an ammonium sulphate fraction with 1 mg. of trypsin in a volume of 1.1 ml. for 15 min. resulted in a complete loss of activity.

Inhibitors

Sodium fluoride at a concentration inhibitory to phosphatase (15 mM) did not inhibit fructokinase. However, higher concentrations proved to decrease the activity. This was demonstrated using phosphatase-free DEAE-cellulose eluates as source of the ATP-dependent enzyme. The effect of NaF on poly-P fructokinase was tested with an ammonium sulphate and a Sephadex G-100 fraction. The results of experiments with NaF, iodoacetate and zinc sulphate are summarized in Table 3.

Table 3

Inhibition of fructokinase activity

ATP fructokinase was assayed using 0.5 ml. of DEAE-cellulose eluate under standard conditions in a total volume of 1.4 ml. Poly-P fructokinase was assayed using either ammonium sulphate fraction I or Sephadex G-100 eluate in which case the reaction mixture with $ZnSO_4$ was adjusted to pH 7.4.

Inhibitor	Concn. (mM)	Activity (%)	
		with ATP	with poly-P
None		100	100
NaF	15	100	
NaF	36	85	80
NaF	72	11	
Iodoacetate	10		87
$ZnSO_4$	1		13
$ZnSO_4$	10		0

DISCUSSION

Fructose-grown *M. phlei* was shown to possess the ability to phosphorylate fructose with ATP, and to a lesser degree with inorganic polyphosphates. The rate of phosphorylation in cell-free extracts was low, a certain amount of enzyme remained, however, unextracted. The relatively poor activity of the extracts could also

be due to considerable lability of the enzymes studied and perhaps to inadequacy of either disintegration or assay despite the attempts to ensure optimum experimental conditions. It is of interest to note that extracts from glucose-grown cells were practically devoid of fructokinase activity.

Taking into account the presence of inorganic poly-P glucokinase in *M. phlei* [20], particular attention was paid to the phosphorylation of fructose with poly-P. Unfortunately, attempts to purify the poly-P-dependent enzyme were largely unsuccessful, the only positive steps being the ammonium sulphate fractionation and Sephadex gel filtration. Contrary to the ATP fructokinase which was recovered from DEAE-cellulose column in good yield, the activity toward poly-P could not be detected in any of the fractions. This suggests that poly-P is not attacked by the enzyme which utilizes ATP. The elution pattern of Sephadex gel filtration, namely the shift of peaks (and in consequence the variable ATP/poly-P activity ratio) may also be taken as evidence for non-identity of the poly-P and ATP fructokinases.

If this is the case, the question would arise whether the phosphorylation of fructose with poly-P resulted from consecutive action of AMP-polyphosphate phosphotransferase, adenylate kinase and ATP fructokinase. Experimental data lend no support for such a three-step mechanism. Dialysis with subsequent passage of enzyme preparations through long Sephadex G-25 column, performed to remove free nucleotides, did not decrease the rate of fructose phosphorylation with poly-P; the addition of either AMP or ADP in catalytic amount had practically no effect on the reaction and, moreover, the optimum pH value for the poly-P fructokinase activity was at pH 8.5, whereas AMP-polyphosphate phosphotransferase was found to be most active at pH 6.5 [4].

It seems then that there are in *M. phlei* two enzymes directly phosphorylating fructose in analogy to those phosphorylating glucose. Occurrence of two different enzymes which catalyse the formation of the same product has been reported in the literature and is understood in terms of cell regulatory mechanisms. As far as the mycobacterial hexokinases are concerned, further research is necessary to elucidate their role *in vivo*.

By Sephadex G-100 gel filtration the fructokinases were separated from glucokinases. The results were clear-cut and reproducible; glucokinases always appeared first with the bulk of other proteins, while fructokinases emerged later. This is indicative of smaller molecular weights of the latter enzymes (below 100 000). In addition to different elution patterns, the fructokinases differ from glucokinases in that they are more labile to heat. On the other hand, the glucokinases were largely inactivated during corund disintegration whereas the fructokinases remained virtually unaffected.

In contrast to specific mammalian ATP fructokinases which produce fructose-1--phosphate [2], those from *M. phlei* phosphorylate fructose at C-6 position. In this respect they resemble the fructokinases of *Pseudomonas saccharophila* [17] and *Alcaligenes faecalis* [5].

Mycobacterial phosphofructokinase deserves particular attention. Bastarrachea *et al.* [1] obtained this enzyme in a partially purified state from *M. tuber-*

culosis H_{37} Ra. We found it in Sephadex eluates from *M. phlei*. However, we were not able to detect any phosphorylation of fructose-6-phosphate at the expense of inorganic poly-P, despite the use of various enzyme preparations both from *M. tuberculosis H_{37} Ra* and *M. phlei*. These negative results may mean that such a poly-P phosphofructokinase does not exist at all, or did not accompany the ATP-dependent enzyme in the preparations tested. The lack of poly-P phosphofructokinase and the presence of poly-P hexokinases may be of importance for the regulation of carbohydrate metabolism in mycobacteria.

REFERENCES

[1] Bastarrachea F., Anderson D. G. & Goldman D. S. - *J. Bact.* **82**, 94, 1961.
[2] Crane R. K. & Sols A., in *Methods in Enzymology*, (S. P. Colowick & N. O. Kaplan, eds.), vol. 1, 277, Academic Press, New York 1955.
[3] Dawson C. R. & Magee R. J., in *Methods in Enzymology*, (S. P. Colowick & N. O. Kaplan, eds.), vol. 2, 823, Academic Press, New York 1955.
[4] Dirheimer G. & Ebel J. P. - *C. R. Acad. Sci. Paris*, **260**, 3787, 1965.
[5] Domagk G. F. & Horecker B. L. - *Arch. Biochem. Biophys.* **109**, 342, 1965.
[6] Doudoroff M., Palleroni J. N., MacGee J. & Ohara M. - *J. Bact.* **71**, 196, 1956.
[7] Fiske C. H. & Subbarow Y. - *J. Biol. Chem.* **66**, 375, 1925.
[8] Forsyth W. G. C. - *Nature* **161**, 239, 1948.
[9] Głębicki T., Jałowiecka K. & Sym E. A. - *Gruźlica* **18**, 413, 1950.
[10] Hanes C. S. & Isherwood F. A. - *Nature* **164**, 1107, 1949.
[11] Kalckar H. M. - *J. Biol. Chem.* **167**, 461, 1947.
[12] Keilin D. & Hartree E. F., in *Methods in Enzymology*, (S. P. Colowick & N. O. Kaplan, eds.), vol. 1, 98, Academic Press, New York 1955.
[13] Kornberg A. & Horecker B. L., in *Methods in Enzymology*, (S. P. Colowick & N. O. Kaplan eds.), vol. 1, 323, Academic Press, New York 1955.
[14] Lowry O., Rosebrough N. J., Farr A. L. & Randall R. V. - *J. Biol. Chem.* **193**, 265, 1951.
[15] McIlwain H. - *J. Gen. Microbiol.* **2**, 288, 1948.
[16] Mokrasch L. C. - *J. Biol. Chem.* **208**, 55, 1954.
[17] Palleroni J. N., Contopoulou R. & Doudoroff M. - *J. Bact.* **71**, 202, 1956.
[18] Roe J. H. - *J. Biol. Chem.* **107**, 15, 1934.
[19] Szymona M. - *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **4**, 121, 1956.
[20] Szymona M. - *Acta Biochim. Polon.* **9**, 165, 1962.
[21] Szymona M. & Ostrowski W. - *Biochim. Biophys. Acta* **85**, 283, 1964.
[22] Trevelyan W. E., Proctor D. P. & Harrison J. S. - *Nature* **166**, 444, 1950.
[23] Trucco R. E., Caputto R., Leloir L. F. & Mittelman N. - *Arch. Biochem.* **18**, 137, 1948.
[24] Umbreit W. W., Burris R. H. & Stauffer J. E., *Manometric Techniques*, Burgess Publishing Co., Minneapolis 1957.

ATP-OWA I POLIFOSFORANOWA FRUKTOKINAZA *MYCOBACTERIUM PHLEI*

Streszczenie

1. W ekstraktach z *M. phlei* rosnących na fruktozie stwierdzono występowanie układu fosforylującego fruktozę na C-6 przy użyciu ATP lub nieorganicznego polifosforanu (sól Grahama). Fruktokinazy oczyszczono 15-krotnie i przez filtrację na Sephadeksie oddzielono od specyficznych glukokinaz.

2. Otrzymany preparat był termolabilny i ulegał całkowitej inaktywacji podczas 1 minutowego ogrzewania w 70°. Reakcja fruktokinazowa wymaga jonów Mg^{2+} i przebiega najszybciej w obecności KCl (0.2 M) przy pH ok. 8.5.

3. Dane doświadczalne przemawiają za tym, że polifosforan jest zużytkowywany przez enzym różny od ATP fruktokinazy.

4. W przeciwieństwie do mykobakteryjnych gluko- i fruktokinaz, preparat fosfofruktokinazy otrzymany z *M. phlei* wykazywał aktywność tylko względem ATP.

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INCORPORATION OF ^{14}C-LABELLED AMINO ACIDS INTO ACID PHOSPHOMONOESTERASE IN SLICES OF HUMAN PROSTATE GLAND

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1. Slices of hypertrophic human prostate gland incubated in Krebs-Ringer solution in the presence of nutrient substrates incorporated ^{14}C-labelled amino acids into the soluble proteins. 2. The incorporation was inhibited in the presence of 2,4-dinitrophenol and under anaerobic conditions. Glucose diminished the incorporation of [^{14}C]leucine by over 50%. 3. A method for small scale isolation of radioactive phosphomonoesterase from the soluble protein fraction of the prostate gland is described.

Preparation of pure proteins labelled with isotopes plays an important role in studies on the mechanism of biosynthesis, metabolism and structure of proteins. By active incorporation of labelled amino acids, radioactive insulin [7, 8], ribonuclease [4, 9], chymotrypsinogen [6], ACTH [1], haemoglobin [13], carbamoylphosphate synthase [15] and other proteins have been obtained.

In the hypertrophic prostate gland marked activity of acid phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) is present and the enzyme can be isolated in the pure state [10]. The enzyme is easily extracted from tissue slices with water [3], which indicates that the enzyme synthesized in the microsomes is quickly excreted into the soluble cell fraction.

In this paper the conditions of incorporation of ^{14}C-labelled amino acids into the soluble protein fraction of the human hypertrophic prostate gland, and isolation from this fraction of highly purified radioactive acid phosphomonoesterase, are reported. Preliminary communications have been presented [14, 11].

MATERIAL AND METHODS

Slices and homogenate. Hypertrophic prostate glands removed during operation were cooled on ice, washed with cold physiological saline solution, and the connective tissue capsule was removed. The glands were cut by hand in the cold room into slices 0.3 - 0.5 mm. thick, which were immediately placed in cooled incubation fluid.

The homogenate was prepared from slices by homogenization in a Waring-Blendor with the incubation fluid, then centrifuged for 5 min. at 2000 rev./min. and the supernatant used for incubation.

Radioactive amino acids. [U-^{14}C]Glycine (160 mc/g.) and D,L-[U-^{14}C]leucine (5.89 mc/g.) were received from the Institute for Nuclear Research (Warszawa, Poland); L-[U-^{14}C]phenylalanine (10.3 mc/m-mole) was from the Radiochemical Centre (Amersham, England).

Incubation medium. Krebs-Ringer solution [5] containing fumarate, glutamate, pyruvate and 0.1 M-phosphate buffer of pH 7.4 without glucose served as standard incubation medium. The solution was supplemented by acid casein hydrolysate (Difco, U.S.A.) to the final concentration of 0.045%. The incubation medium was gassed with a mixture of O_2/CO_2 (95 : 5, by vol.), then 1.0 - 1.5 μc of ^{14}C-labelled amino acid per gram of tissue was added. In some experiments the standard incubation medium was modified by adding glucose, or omitting the mixture of fumarate, pyruvate and glutamate, or by replacing the oxygen atmosphere with nitrogen.

Conditions of incubation. The incubation was carried out in Warburg vessels or in 50-ml. Erlenmayer flasks in an atmosphere of O_2/CO_2 at 37° with a fivefold volume of the incubation medium in relation to the weight of the slices. The maximum weight of the tissue in Warburg vessels was 1 g., and in the Erlenmayer flasks 10 g. During the incubation the vessels were shaken at a rate of 60 oscillations/min.

Preparation of protein for radioactivity estimations. After a determined time of incubation, 10 μmoles of appropriate unlabelled amino acid per gram of the tissue was added and the whole cooled in ice. The slices were then homogenized in the Waring-Blendor in the same incubation medium, centrifuged for 5 min. at 2000 rev./min., and the proteins present in the supernatant were fractionated with ammonium sulphate. The fraction precipitated at 52 - 68% saturation, which contained most of the acid phosphomonoesterase activity of the extract [12], was collected, dissolved in a small amount of water, and an equal volume of 10% trichloroacetic acid was added. After centrifuging, the precipitate was suspended in 5% trichloroacetic acid, heated for 15 min. at 90° and centrifuged. Then the precipitate was washed successively with 5% trichloroacetic acid, 95% ethanol, ethanol - ether mixture (3 : 1, v/v) and finally with ether. The protein was dissolved in formic acid, transferred to an aluminium planchet and dried under an infrared lamp. The radioactivity was measured in a gas-flow counter, 2Pi, Frieseke-Hoepfner Co. (Erlangen, West Germany), under conditions similar to those used for counting at infinite thinness, so that the loss of activity due to self-absorption was negligible.

Isolation of radioactive phosphomonoesterase. For the isolation of the enzyme, about 50 g. of slices incubated in several flasks under standard conditions for 2 hr., was homogenized in a Waring-Blendor for 45 sec. After centrifuging, the sediment was discarded, and the supernatant dialysed against water for 24 hr. and fractionated with ammonium sulphate. All the procedures for isolating the enzyme were carried out in the cold room at about 2°. The fraction precipitated at 52 - 68% saturation was isolated by centrifuging and extracted with 10 volumes of 0.1 M-citric acid - NaOH buffer, pH 4.0. The suspension was centrifuged at 20 000 *g* and the supernatant was

dialysed against 0.0175 M-Na-phosphate buffer of pH 7.0 and applied on DEAE--cellulose column (1.8×25 cm.) equilibrated with the same buffer. After washing the column with the same buffer, the elution was carried out with Na-phosphate buffer, pH 5.5, as described previously [12]. The enzymically active fractions eluted with 0.05 M buffer (Fig. 2) were pooled, dialysed against 0.0175 M-Na-phosphate buffer, pH 7.0, and frozen at -20°. The preparations from two 50-g. portions of slices were combined and applied on the DEAE-cellulose column equilibrated with the 0.0175 M-Na-phosphate buffer, pH 7.0. The column was washed with about 300 ml. of the same buffer, and the enzyme was eluted by a pH and ionic strength gradient. The mixing chamber contained 250 ml. of 0.0175 M-Na-phosphate buffer, pH 7.0, and the reservoir 0.07 M-Na-phosphate buffer, pH 6.0; 7-ml. fractions were collected and examined for the presence of protein by measuring the extinction at 280 mμ, for enzymic activity, and for radioactivity which was measured in 0.5-ml. portions evaporated on aluminium planchet, as described above. The enzymically active fractions were pooled, dialysed against 0.01 M McIlvaine buffer (citric acid - Na-phosphate), and rechromatographed on the CM-cellulose column (1.8×22 cm.). Elution was carried out with a pH gradient from 4 to 5. The mixing chamber contained 500 ml. of 0.01 M McIlvaine buffer, pH 4.0, and the reservoir 0.02 M McIlvaine buffer, pH 5.0; 8-ml. fractions were collected. In this way nearly homogeneous enzyme was obtained.

Estimation of enzymic activity. Acid phosphomonoesterase activity was estimated using as substrate 0.02 M-sodium *p*-nitrophenylphosphate (Sigma, Biochem. Corp., St. Louis, U.S.A.) dissolved in 0.1 M-citric acid - NaOH buffer of pH 5.0 [3]. To 200 μl. of substrate solution, 2 - 10 μl. of enzyme solution was added by means of a micropipette (Hamilton, Whittier, U.S.A.), and the mixture was incubated at 25° for 60 sec. The reaction was stopped by adding 2.8 ml. of 0.1 M-NaOH, and the liberated *p*-nitrophenol determined at 400 mμ in an Uvispec (Hilger & Watts, London) spectrophotometer. The enzyme activity was expressed as μmoles of *p*-nitrophenol liberated by 1 ml. of enzyme solution at 25° during 1 min.

RESULTS

Radioactive glycine, leucine and phenylalanine were rapidly incorporated into the soluble proteins of the human prostate gland. The greatest incorporation was observed when the slices were incubated in an atmosphere of oxygen and in the absence of glucose in the medium. The relation of incorporation of [^{14}C]glycine to time of incubation, under these conditions, is illustrated in Fig. 1. Presence in the incubation medium of fumarate, pyruvate and glutamate enhanced the incorporation of [^{14}C]glycine and [^{14}C]leucine about twofold compared with the conditions in which glucose was the only source of energy (Table 1). At 0.01 M-glucose concentration the radioactivity of protein was about one-half that of the sample incubated in the presence of fumarate, pyruvate and glutamate as the source of energy.

The possibility of adsorption or non-specific exchange of amino acids was tested by carrying out the incubation under anaerobic conditions. In the atmosphere

of N_2/CO_2 the incorporation of [^{14}C]leucine was inhibited by 70%, indicating that approximately this part of radioactivity is due to active synthesis of protein in the gland slices, since the ability to adsorb or exchange amino acids in the soluble protein fraction should be the same both under anaerobic and aerobic conditions. The degree of adsorption and/or exchange was determined also by another method. The soluble protein, ammonium sulphate fraction obtained from slices which had been incubated in the presence of [^{14}C]leucine, was dialysed for 48 hr. against a

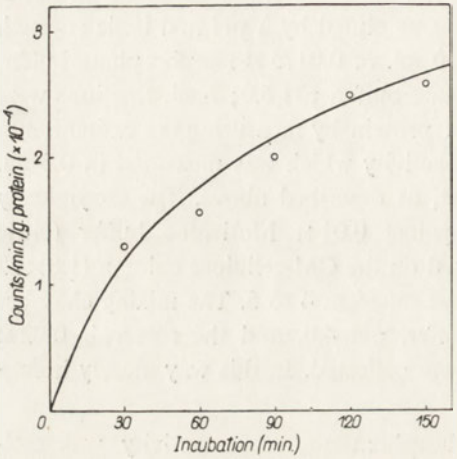

Fig. 1. Rate of incorporation of [U-^{14}C]glycine into the soluble protein fraction of slices of the human prostate gland. Radioactivity of the ammonium sulphate 52 - 68% sat. fraction was measured.

20 mM-solution of unlabelled leucine, and the specific activities of the protein before and after dialysis were compared. It was found that after dialysis only a slight decrease (about 10%) in the activity occurred, i.e. about 90% of the activity was due to active incorporation. Similar results were obtained when 0.2 mM-2,4-dini-

Table 1

The effect of conditions of incubation on the incorporation of [^{14}C]*leucine into soluble proteins of the hypertrophic human prostate gland*

One gram of slices or a corresponding amount of homogenate was incubated for 30 min. at 37° in Krebs-Ringer II solution, with acid casein hydrolysate and 1.5 μc of D, L-[^{14}C]leucine added. The standard incubation conditions were modified as shown in the Table. The results are expressed in relation to those obtained under standard conditions, taken as 100. Each value is a mean from 3 samples. Radioactivity was determined in the soluble protein (52 - 68% ammonium sulphate sat. fraction).

Incubation conditions	Radioactivity
Slices, complete system	100
fumarate, pyruvate and glutamate omitted	46.5
0.01 M-glucose added	42.6
in N_2 atmosphere	30
0.2 mM-2,4-dinitrophenol added	20
complete system, soluble protein fraction dialysed for 48 hr.	90
Homogenate, complete system	16.5

trophenol was added to the incubation medium. The uncoupling of oxidative phosphorylation inhibited the incorporation of labelled amino acids into the soluble protein fraction by over 80% (Table 1).

Disintegration of the cellular structure by homogenization resulted in practically complete inhibition of [^{14}C]leucine incorporation. Radioactivity of the samples measured at zero-time incubation was the same in all experiments, not exceeding several counts/min./mg. of protein.

Incorporation of [^{14}C]*phenylalanine into the enzyme molecule.* About 50 g. of slices was incubated in 10-g. portions in the standard incubation medium under aerobic conditions with 10 μc of [^{14}C]phenylalanine per gram of tissue in 50-ml. Erlenmayer flasks. After 2-hr. incubation, the slices were homogenized, and the enzyme was isolated and purified as described in Material and Methods. Figure 2 illustrates the first chromatographic separation of soluble prostate proteins on

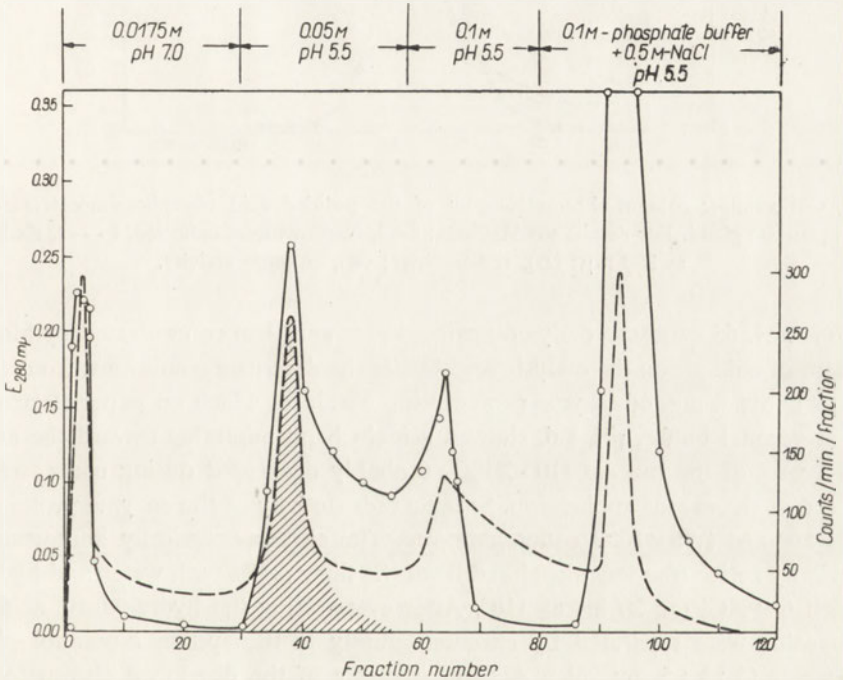

Fig. 2. Stepwise chromatographic separation on the DEAE-cellulose column of proteins obtained after incubation of 10 g. of prostate slices with [U-^{14}C]phenylalanine. For details see Methods. Five-ml. fractions were eluted at a rate of about 60 ml./hr. (- - -), Extinction at 280 mμ; (○), radioactivity. The hatched area represents the range of acid phosphomonoesterase activity.

the DEAE-cellulose column. The radioactive peak eluted with 0.05 M-phosphate buffer, pH 5.5, contained more than 70% of the phosphomonoesterase present in the adsorbed sample. The highest radioactivity was found in the fraction eluted with 0.1 M-phosphate buffer of pH 5.5 containing 0.5 M-NaCl, i.e. the fraction of acid proteins with strong affinity for the adsorbent.

The enzymically active peak was rechromatographed twice as described in Methods. The active peak eluted from the CM-cellulose column showed coincidence of radioactivity, enzymic activity and extinction at 280 mμ (Fig. 3). The pooled

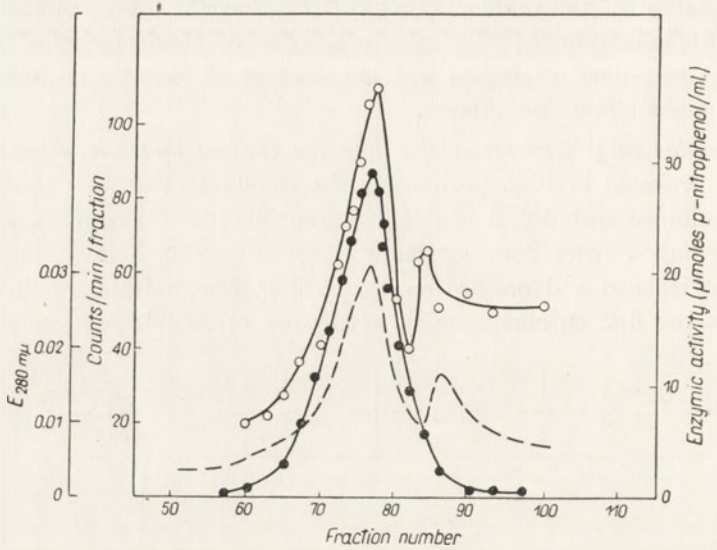

Fig. 3. CM-cellulose column chromatography of the purified acid phosphomonoesterase from human prostate gland. For details see Methods; 8-ml. fractions were collected. (- - -), Extinction at 280 mμ; (○), radioactivity; (●), enzymic activity.

fractions nos. 65 - 80 were dialysed against water and then concentrated by blowing a current of cold air on the dialysis bag. Under the described conditions, from 100 g. of slices about 4 mg. of enzyme protein were obtained, which on paper electrophoresis in veronal buffer, pH 8.6, showed a main band migrating toward the anode, while a part of the protein (10 - 20%), probably denatured during concentration, remained at the site of application. Specific radioactivity of the enzyme protein was on the average 1.75×10^{-2} counts/min./mg. The electrophoretically homogeneous, enzymically active fraction was eluted from the filter paper with water and hydrolysed with 6 N-HCl for 20 hr. at 110°. After evaporating the hydrochloric acid, the amino acids were separated by chromatography in the system *n*-butanol - acetic acid - water (4 : 1 : 5, by vol.). Autoradiography of the developed chromatogram showed that the R_F value of the radioactive spot corresponds to that of the ninhydrin spot of phenylalanine used as standard.

DISCUSSION

The described experiments showed that slices of hypertrophic human prostate gland *in vitro* incorporate amino acids into soluble proteins. The incorporation of ^{14}C-labelled amino acids into protein was not due to adsorption or exchange of amino acids; it was dependent on energy supply and was inhibited by uncoupling of oxidative phosphorylation or by anaerobic conditions. The incorporation of

[^{14}C]leucine was distinctly diminished when the incubation medium containing fumarate, pyruvate and glumate, was supplemented with glucose, or contained only glucose as energy donor. Two explanations of this phenomenon can be suggested: (a), glucose inhibits protein synthesis in human prostate; (b), a considerable amount of ATP is utilized within the cell for the phosphorylation of glucose, leading to a reduction of synthesis of active amino acids. Diminished incorporation of amino acids into proteins in the presence of glucose has been observed also by Bauer & Lazarow [2].

Although the net synthesis of acid phosphomonoesterase has not been demonstrated, the experiments described above speak against a non-specific association of [^{14}C]phenylalanine with the enzyme. The specific radioactivity of the isolated enzyme was relatively low owing to the low activity of the amino acid used. Therefore it was not possible to carry out a detailed analysis of the radioactivity of the peptides obtained by tryptic digestion of phosphomonoesterase and to demonstrate a *de novo* synthesis of the whole enzyme molecule. However, after complete acid hydrolysis of the purified enzyme, radioactive phenylalanine was isolated.

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REFERENCES

[1] Adiga P. R., Uemura I. & Winnick T. - *Biochemistry* **4**, 246, 1965.

[2] Bauer G. E. & Lazarow A. - *Biol. Bull.* **121**, 425, 1961.

[3] Boman H. G. - *Arkiv Kemi* **12**, 453, 1958.

[4] Craddock V. M. & Dalgliesh C. E. - *Biochem. J.* **66**, 250, 1957.

[5] Dawson R. M. C., Elliott D. C., Elliott W. H. & Jones K. M., *Data for Biochemical Research*, p. 208. Clarendon Press, Oxford 1959.

[6] Farinha J., Gundlach G. & Turba F. - *Biochem. Z.* **339**, 543, 1964.

[7] Light A. & Simpson M. V. - *Biochim. Biophys. Acta* **20**, 251, 1956.

[8] Mallory A., Smith G. H. & Taylor K. W. - *Biochem. J.* **91**, 484, 1964.

[9] Morris A. J. & Dickman S. R. - *J. Biol. Chem.* **235**, 1404, 1960.

[10] Ostrowski W. & Rybarska J. - *Biochim. Biophys. Acta* **105**, 196, 1965.

[11] Ostrowski W., Rybarska J. & Koj A., 2nd *Meeting Abstr. Federation Europ. Biochem. Soc., Vienna*, 1965, p. 4.

[12] Ostrowski W. & Tsugita A. - *Arch. Biochem. Biophys.* **94**, 68, 1961.

[13] Rabinovitz M. & Fisher J. M. - *Biochim. Biophys. Acta* **91**, 313, 1964.

[14] Rybarska J. & Ostrowski W., *XXII Congress of Polish Radiologists, Kraków*, 1964, p. 15.

[15] Tatibana M. & Cohen P. P. - *J. Biol. Chem.* **239**, 2905, 1964.

INKORPORACJA ^{14}C-ZNAKOWANYCH AMINOKWASÓW DO FOSFOMONOESTERAZY SKRAWKÓW GRUCZOŁU KROKOWEGO

Streszczenie

1. Wykazano, że inkubowanie skrawków przerosłego ludzkiego gruczołu krokowego w płynie Krebsa-Ringera w obecności substratów odżywczych prowadzi do inkorporacji ^{14}C-znakowanych aminokwasów do białek rozpuszczalnych.

2. Inkorporacja jest wyraźnie zahamowana przez obecność 2,4-dwunitrofenolu oraz przez inkubację skrawków w warunkach beztlenowych. Obecność glikozy w środowisku inkubacyjnym powoduje zmniejszenie inkorporacji [^{14}C]leucyny o ponad 50%.

3. Opisano warunki izolowania na małą skalę radioaktywnej kwaśnej fosfomonoesterazy z frakcji białek rozpuszczalnych sterczu.

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PARTIAL REVERSAL BY PURINE AND PYRIMIDINE BASES OF YEAST GROWTH INHIBITION PRODUCED BY 3-AMINO-1,2,4-TRIAZOLE

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1. Growth of the wild strain of *Saccharomyces cerevisiae* inhibited by low concentrations (less than 2 mM) of 3-amino-1,2,4-triazole (3-AT), is limited by adenine synthesis. At high 3-AT concentration (20 mM), histidine biosynthesis becomes the growth-limiting process. 2. Experiments on hi_8 mutant of *S. cerevisiae* indicated that, in addition to imidazoleglycerolphosphate dehydratase, another enzyme required for the synthesis of adenine and cytosine is also 3-AT sensitive.

3-Amino-1,2,4-triazole (3-AT) produces a transient inhibition of growth of *Saccharomyces cerevisiae* cells; an adaptation process readily renders them almost insensitive to the inhibitor. L-Histidine is able to prevent completely both the growth inhibition by moderate concentrations of 3-AT and the adaptation process [11]. The effect of histidine on yeast growth is due to the fact that 3-AT is a strong competitive inhibitor of imidazoleglycerolphosphate (IGP) dehydratase (EC 4.2.1.19), an enzyme of histidine biosynthesis, both *in vitro* [14] and *in vivo*, as evidenced by imidazoleglycerol (IG) accumulation in culture media [12].

However, in addition to histidine, several other natural compounds are able to release, but only partially, the yeast growth from the inhibition by 3-AT. As shown by Hilton [7], the most effective were cytosine, guanine and adenine. The same author reported that also some L-amino acids were capable of partial reversal of the inhibition by 3-AT, namely leucine, tyrosine, methionine, isoleucine and tryptophan.

In other organisms, like *Escherichia coli* [17] and *Salmonella typhimurium* [10] full reversal of 3-AT inhibited growth can be achieved only by the addition of both histidine and adenine. Either of these compounds applied alone has but a partial effect. IGP dehydratase in *Salmonella typhimurium* has been shown to be sensitive to 3-AT [10].

The present results support the concept of Hilton, Kearney & Ames [10] that there is a second point of attack of 3-AT. Most probably, 3-AT affects the availability of a compound utilized by yeast for the biosynthesis of adenine and cytosine. Preliminary results of this work have been presented [4].

METHODS

Organisms. All strains used were kindly supplied by Dr. M. Luzzati, Gif-sur--Yvette, France. The two prototrophic diploids of *Saccharomyces cerevisiae*, YF and LM 2, and the heteroallelic ad_3 mutant LM 27, requiring histidine and adenine, were described previously [11]. The histidine-requiring haploid mutant 198-4D (derived from Sherman's strain S1237B) bears a mutation of gene hi_8, which controls IGP dehydratase similarly as hi_3 gene (G.R. Fink, personal communication).

Media, cultures and growth tests were as described previously [11]. The minimal liquid and solid media of Galzy & Slonimski [6] were used throughout.

Chemical assay. IGP and IG were determined by the method of Ames [1], as described in [12].

Enzyme assay. IGP dehydratase activity was determined by the method of Ames [1] adapted for the enzyme of *S. cerevisiae* [14].

Chemicals. IGP was synthesized according to Ames [1], as outlined in [14]. All other chemicals were commercial products. 3-Amino-1,2,4-triazole was purchased from the California Corporation for Biochemical Research (Los Angeles, U.S.A.), Fluka AG (Buchs, Switzerland) and Serlabo (Paris, France). In spite of apparently different purity, all three 3-AT preparations had the same effect on yeast cells or on the purified IGP dehydratase, which indicates that 3-AT itself and not any impurity present in the preparations, was responsible for the effects observed. Purine and pyrimidine deoxyribosides and ribosides, thymine and 5-aminoimidazole-4--carboxamide hydrochloride were products of the California Corporation for Biochemical Research (Los Angeles, U.S.A.). Other chemicals used were: L-histidine hydrochloride monohydrate (Chemapol, Prague, Czechoslovakia), cytosine and uracil (British Drug Houses Ltd., Poole, England), orotic acid (Nutritional Biochemicals Corp., Cleveland, U.S.A.), hypoxanthine and guanine (Light & Co. Ltd., Colnbrook, England), adenine (Feinchemie, Berlin, Germany).

RESULTS

Effect of purines, pyrimidines and their derivatives on yeast growth in the presence of 1 mM-3-*AT*

The effect of 1 mM-3-AT on *S. cerevisiae* can be fully prevented by histidine [11, 12]. The experiments shown in Fig. 1 demonstrate that purine and pyrimidine bases (excepting thymine) accelerate the appearance of the phase of active growth of yeast cells in the presence of 1 mM-3-AT; adenine and cytosine were the most effective. The effect of guanine was hard to evaluate because of its poor solubility in the culture medium. Hypoxanthine and uracil were significantly less effective. These results are in agreement with those of Hilton [7]. Deoxyribosides of guanine, adenine and cytosine (Fig. 2) had also a growth-promoting effect on the 3-AT inhibited yeast, although the riboside derivatives were inactive. The activity of the deoxyribosides could be due to their hydrolysis in the acid (pH 4) culture medium.

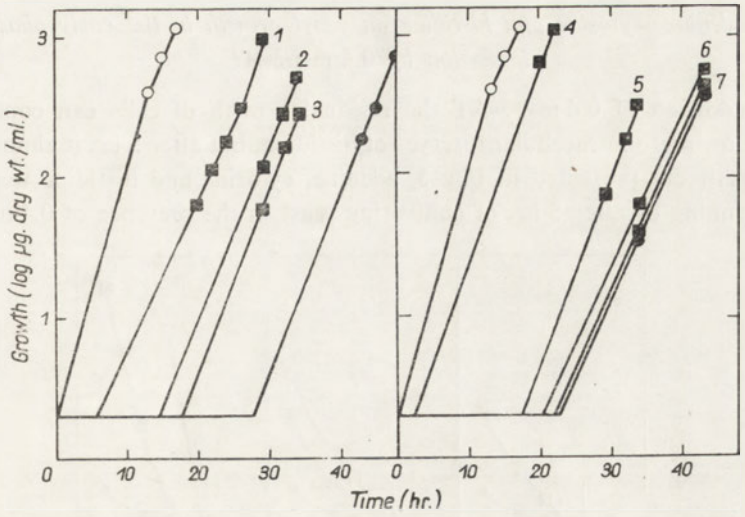

Fig. 1. Effect of purine and pyrimidine bases on the growth of *S. cerevisiae* YF strain inhibited by 3-AT. (○), Control, without 3-AT; (●), with 1 mM-3-AT; (■), with 1 mM-3-AT and: (*1*), 0.1 mM-adenine; (2), 0.1 mM-hypoxanthine; (*3*), 0.1 mM-guanine; (*4*), 1 mM-cytosine; (*5*), 1 mM--uracil; (*6*), 1 mM-thymine; (*7*), 1 mM-orotic acid. In the early phase of growth, cell density was too low to be measured. That is why the lower parts of the growth curves which were obtained by extrapolating the upper parts to zero-time cell density, only approximately represent the growth.

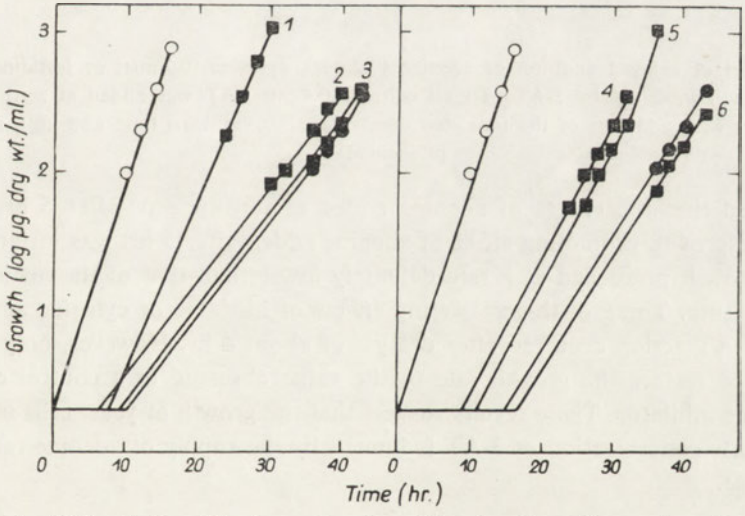

Fig. 2. Effect of (*1*), adenine; (2), deoxyadenosine; (*3*), adenosine; (*4*), guanine; (*5*), deoxyguanosine and (6), guanosine, added to the final concn. of 0.1 mM, on YF growth inhibited by 3-AT. Other conditions as in Fig. 1. (○) Control; (●), with 1 mM-3-AT.

The two tested precursors of purines and pyrimidines, 5-amino-4-imidazolecarboxamide and orotic acid were inactive, probably because of inability to penetrate into the yeast cells.

Effect of adenine, cytosine and histidine on yeast growth in the early phase of inhibition by 0.4 mM-3-*AT*

After addition of 0.4 mM-3-AT the residual growth of cells can continue for some time owing to intracellular reserves of histidine, but after 5 hr. it almost ceases. In the experiment presented in Fig. 3, adenine, cytosine and histidine were added at the beginning or after 5 hr. of cultivating yeast in the presence of 0.4 mM-3-AT.

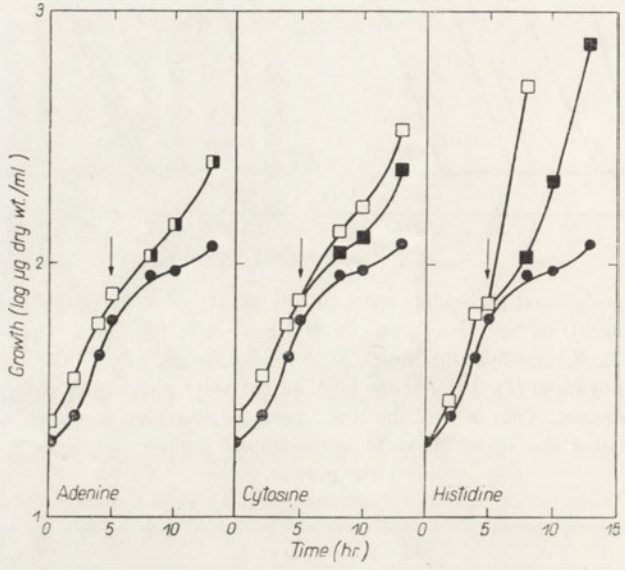

Fig. 3. Effect of delayed addition of adenine (0.2 mM), cytosine (0.2 mM) or histidine (0.1 mM) on YF growth inhibited by 3-AT. To all cultures 0.4 mM-3-AT was added at zero time. (●), 3-AT alone; with addition of the indicated compound (□), at zero time and (■), after 5 hr. of incubation.

It appeared that the effects of adenine added at zero-time or after 5 hr. did not differ. The growth-promoting effect of adenine added after 5 hr. was instantaneous, but the growth proceeded at a rate definitely lower than that of the histidine-containing culture. The growth-accelerating effects of histidine or cytosine added after 5 hr. of 3-AT action occurred after delays of about 4 hr. However, only histidine was able to restore the growth rate to the value observed in a control cultivated without the inhibitor. These results suggest that the growth of yeast cells in the presence of low concentration of 3-AT is limited by the supply of adenine rather than of histidine.

Dependence of points of growth deceleration in the presence of 1 mM-3-*AT on the growth-limiting concentrations of adenine*

In the previous paper [12] it has been demonstrated that yeast inhibited by 3-AT behaves like a histidineless mutant. In this work a similar experiment was performed with adenine (Fig. 4A). Cultures of strain YF containing low concentrations of adenine added, resumed growth after a lag phase at a rate intermediate between

those of a culture containing no inhibitor and a culture containing the inhibitor but without the addition of adenine. However, after a time depending on adenine concentration the growth rate slowed down to that of the culture with 3-AT alone. These growth rate decelerations suggest the exhaustion of exogenous adenine. Figure 4B represents an analogous experiment with the adenine-requiring mutant strain LM 27. In this case the growth rate tended to stop. In the culture containing

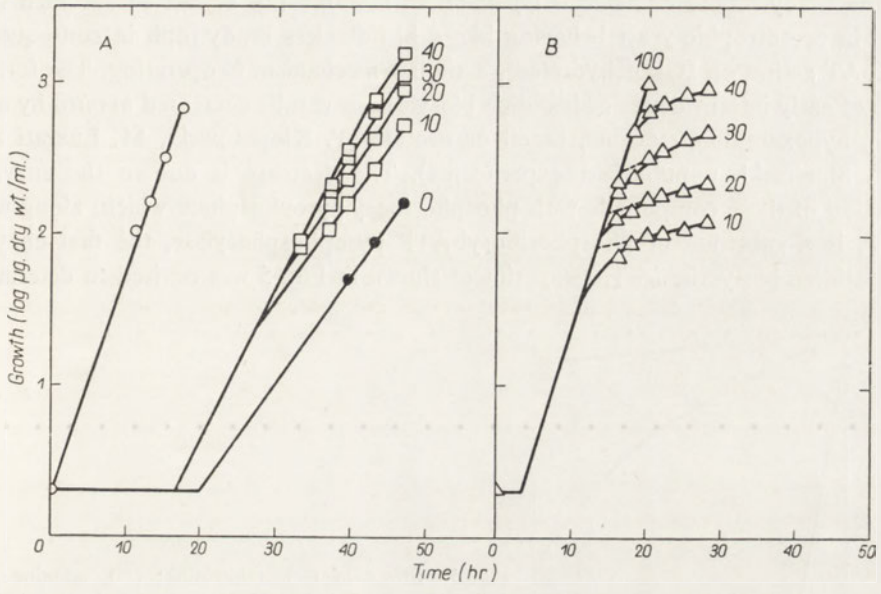

Fig. 4. Adenine as the growth-limiting factor in (*A*), the prototrophic YF strain at 2 mM-3-AT concn., and (*B*), the adenine-histidine auxotroph LM 27 of *S. cerevisiae*. (○), Control, without 3-AT; (●), with 2 mM-3-AT only; (□), with the addition of adenine in concn. indicated, in mμmoles per ml.; (△), with 0.25 mM-L-histidine, and adenine in concn. indicated. The broken lines were obtained by extrapolating the residual growth after exhaustion of adenine. Their points of intersection with the curve for 1 mM-adenine concn. indicate the logarithms of growth yields per amount of adenine present. The average yield was 7.6 mg. dry wt. per 1 μmole adenine.

10 μM-adenine the growth rate was lower by 92% than that with adenine added in excess. In the former case, i.e. YF culture in the presence of 3-AT, the growth rate after the exhaustion of exogenous adenine was inhibited by 76%. Taking into account that the range of effective adenine concentrations was almost the same, it may be concluded that prototrophic yeast exposed to 1 mM-3-AT suffers from adenine shortage and behaves like a bradytrophic adenineless mutant.

The effect of histidine, adenine and cytosine on IGP accumulation produced by 1 mM-3-*AT*

Bradytrophic histidineless mutants of *E. coli* [16] and *Salmonella typhimurium* [2] blocked in early steps of histidine biosynthesis show an alternative requirement for adenine. This phenomenon is due to the fact that all intermediates of histidine

biosynthesis and histidine itself contain two atoms derived from N-1 and C-2 of adenine. In the absence of exogenous histidine, the excessive formation and excretion of the intermediates leads to deficiency of adenine compounds, which become growth-limiting factors. A decrease of the losses of histidine intermediates caused even by otherwise inhibitory agents like 2-thiazolealanine, a false feed-back inhibitor of the first enzyme of histidine biosynthesis, results in increased growth rate of the bradytrophic *Salmonella typhimurium* mutants [2]. It can be assumed that also in prototrophic yeast behaving like a histidineless bradytroph in consequence of 3-AT action on IGP dehydratase, a similar mechanism is operating. The formation of early intermediates of histidine biosynthesis can be decreased *in vitro* by adenine, hypoxanthine, cytosine, uracil or orotate (T. Kłopotowski, M. Luzzati and P. P. Slonimski, unpublished experiments). The decrease is due to the enzymic reaction of these compounds with phosphoribosylpyrophosphate which, along with ATP, is a substrate of phosphoribosyl-ATP pyrophosphorylase, the first enzyme of histidine biosynthesis. The experiment shown in Fig. 5 was devised to determine

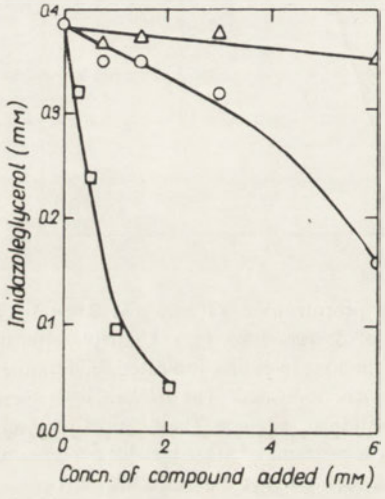

Fig. 5. Effect of (□), L-histidine; (○), adenine and (△), cytosine on imidazoleglycerol excretion caused by 3-AT. The minimal media supplemented with 1 mM-3-AT and different amounts of L-histidine, adenine or cytosine, were inoculated with cells of LM 2 strain of *S. cerevisiae* (300 μg. dry wt./ml.) and incubated for 20 hr. with shaking. Imidazoleglycerol was assayed in media.

whether a similar mechanism may be operating within the living yeast cells. It appeared that in 3-AT inhibited yeast not only histidine, a feed-back inhibitor of phosphoribosyl-ATP pyrophosphorylase, but also adenine decreased the formation of IGP as shown by IG accumulation in culture media. The effect of cytosine, although less marked, was also significant because cytosine promoted the yeast growth under the conditions of the experiment. This result supports the concept that the apparent adenine bradytrophy produced by 3-AT may result from the losses of IG to the medium. However, the experiments to be shown in the following section indicate that another mechanism is also involved.

Second point of action of 3-AT in yeast

Hilton, Kearney & Ames [10] have shown that the relative resistance of *Salmonella typhimurium* to 3-AT is due to prompt derepression of enzymes of histidine biosynthesis. If, however, the concentration of 3-AT is increased to 20 mM

the resulting inhibition of growth cannot be reversed otherwise than by addition of histidine and adenine. The authors suppose that 3-AT is able to inhibit not only IGP dehydratase but also an enzyme involved in the biosynthesis of adenine.

In the present work similar results were obtained with the prototrophic strain YF of *S. cerevisiae*. Histidine alone was unable to reverse completely the effect of 20 mM-3-AT. Full reversal, however, was obtained not only in the presence of histidine and adenine, but also of histidine and cytosine. This suggests that in yeast, similarly as in *Salmonella typhimurium*, there is a second 3-AT sensitive enzyme, the activity of which is required in yeast for the synthesis of both adenine and cytosine.

To distinguish more clearly between the consequences of excessive IGP formation, and the direct interference with adenine and cytosine synthesis, an experiment was carried out on the hi_8 mutant of *S. cerevisiae*. The hi_8 locus is controlling

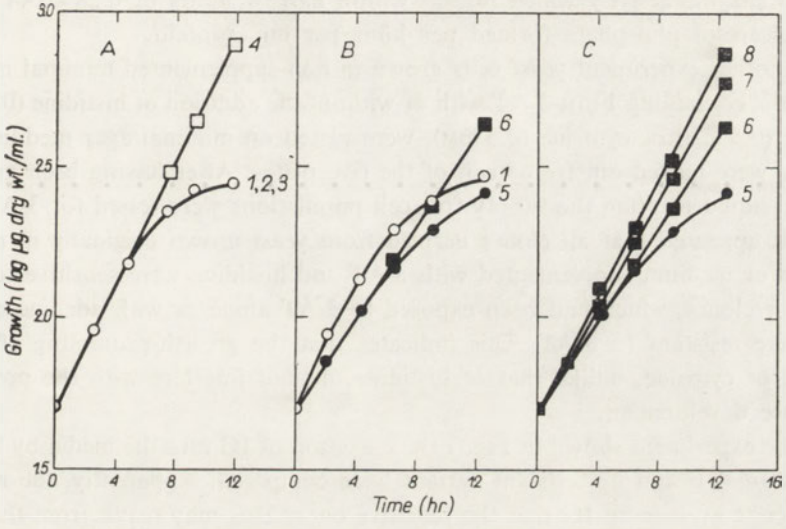

Fig. 6. Effect of adenine, cytosine and histidine on the growth of hi_8 mutant of *S. cerevisiae* in the presence of 20 mM-3-AT. To the minimal medium adenine and cytosine were added to 0.5 mM final concn., and L-histidine to 0.2 mM. (*1*), No additions; (*2*), adenine alone; (*3*), cytosine alone; (*4*), histidine alone; (*5*), 3-AT alone; (6), 3-AT and histidine; (*7*), 3-AT, histidine and cytosine; (*8*), 3-AT, histidine and adenine.

IGP dehydratase, and the respective mutants show very little or no activity of this enzyme and excrete IG into the medium (see Methods). The effect of adenine and cytosine on the growth of the mutant was studied in the presence or absence of histidine and/or 3-AT (Fig. 6). In the absence of 3-AT, in spite of IGP accumulation, adenine and cytosine had no effect on the growth of the mutant, nor had they any effect on its growth in the presence of histidine (Fig. 6A). At 20 mM-3-AT concn. only histidine had a partial reversing effect; the other compounds tested were inactive (Fig. 6B). This means that under the latter conditions, the biosynthesis of histidine is the growth-limiting process. Adenine or cytosine increased the growth rate of the mutant in the presence of 3-AT and histidine almost to the con-

trol value with histidine but without 3-AT. Adenine was repeatedly more effective in this respect than cytosine (Fig. 6C). Taking into account that histidine biosynthesis is the growth-limiting process in yeast exposed to 20 mM-3-AT and that histidine fully prevents the losses of intermediates of its own biosynthesis, it follows from the above data that 3-AT directly affects a process involved in the biosynthesis of adenine and cytosine.

The effect of histidine, adenine and cytosine on the development of resistance to 3-AT

A series of resistant YF yeast populations has been prepared in the presence of seven concentrations of 3-AT ranging from 0.0125 to 0.8 mM, as described earlier [11]. IGP dehydratase activity was determined in cell-free extracts. It appeared that there was no correlation between 3-AT concentration in the adaptation medium and the enzyme activity which ranged within narrow limits of 0.28 - 0.44 μmoles imidazoleacetol phosphate formed per hour per mg. protein.

In another experiment yeast cells grown in non-supplemented minimal medium, and media containing 1 mM-3-AT with or without the addition of histidine (0.25 mM) adenine (0.5 mM) or cytosine (0.5 mM), were plated on minimal agar medium. Five colonies were picked out from each of the five plates. After having been grown in minimal liquid medium the twenty five cell populations were tested for 3-AT resistance. It appeared that all clones derived from yeast grown originally in minimal medium or medium supplemented with 3-AT and histidine were sensitive to 3-AT. All other clones, which had been exposed to 3-AT alone or with adenine or cytosine, were resistant to 3-AT. This indicates that the growth-promoting effects of adenine or cytosine, unlike that of histidine, do not interfere with the process of resistance development.

In the experiment shown in Fig. 7 the excretion of IG into the media by the sensitive strain YF and its resistant variant were compared. Apparently, the resistant cells excrete even more IG that the sensitive ones. This may result from the better growth of the resistant cells during the experiment. However, it may be concluded therefrom that 3-AT inhibits IGP dehydratase within the resistant cells, and hence that the impermeability to 3-AT can be rejected as a mechanism of yeast resistance to the inhibitor.

DISCUSSION

In previous papers evidence has been presented that 3-AT inhibits yeast growth by inhibiting competitively IGP dehydratase [12, 14]. This conclusion has been supported by the results of Hilton & Kearney [9]. In further studies, Hilton, Kearney & Ames [10] found that in *Salmonella typhimurium* 3-AT inhibits, in addition to IGP dehydratase, an enzyme involved in the biosynthesis of adenine. In the present work, interference by 3-AT with this process was observed in *S. cerevisiae*. However, in yeast the ability of both adenine and cytosine to accelerate the growth in the presence of histidine and high concentration of 3-AT suggests that the inhibited

reaction is required for the synthesis both of purine and pyrimidine compounds. The discrepancy between the results of Hilton *et al.* and the present work with respect to the effect of cytosine, may be attributed to the differences between *Salmonella typhimurium* and *S. cerevisiae* since, when the experiments of Hilton *et al.* were repeated (D. Hulanicka & T. Kłopotowski, unpublished experiments) using the wild strain LT-2 of *Salmonella typhimurium* or the histidine-requiring mutant G-70, only adenine and not cytosine was able to accelerate bacterial growth in the presence of 20 mM-3-AT and histidine.

There are no direct links between the pathways of biosynthesis of adenine and cytosine. Probably, the metabolism of a simple compound required for the two processes, is impaired by 3-AT. Some indications as to the identity of this second 3-AT sensitive reaction in yeast are provided by comparison of the effectiveness of aminated purine and pyrimidine bases with those bearing no amino groups.

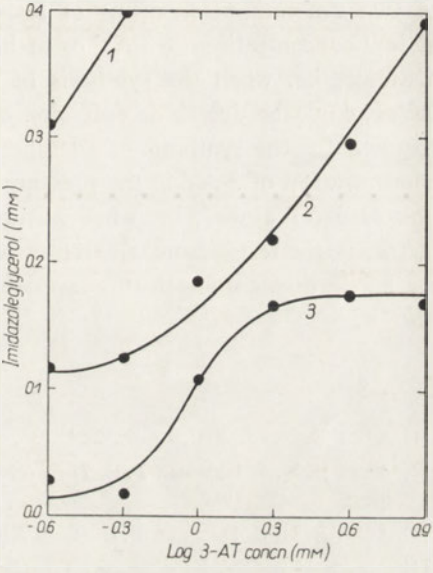

Fig. 7. Effect of 3-AT on the excretion of imidazoleglycerol by 3-AT sensitive and resistant cells of strain YF and by cells of strain LM 2. To the minimal medium only 3-AT was added in concn. ranging from 0.25 to 8.0 mM. (*1*), Strain LM 2; (*2*), 3-AT-resistant YF cells; (*3*), 3-AT-sensitive YF cells.

Since both adenine and cytosine are more effective than hypoxanthine and uracil in reversing the inhibition of yeast growth caused by 3-AT, the interference of this inhibitor with the metabolism of an amino group donor may be suspected. However, aspartate and glutamine, which provide amino groups for the synthesis of both adenine and cytosine, did not influence the growth of yeast inhibited by 3-AT (Hilton [7]). Similarly, in our experiments (unpublished) no effect of aspartate, glutamate, asparagine or glutamine could be detected. The alternative possibility, that 3-AT interferes with the metabolism of the precursor of C-2 atom of adenine seems to be less probable since this atom is provided by transformylation which in turn is not involved in the synthesis of the pyrimidine ring.

Apparently, it may seem paradoxical that at low 3-AT concentrations, when histidine is fully able to prevent the inhibitory effect of 3-AT, adenine biosynthesis is the growth-limiting process whereas at high 3-AT concentrations, when histidine is unable to restore the growth rate to the control value, histidine biosynthesis

limits the growth of yeast cells. This paradox may be explained by assuming that the second 3-AT sensitive reaction is involved in the metabolism of a precursor of one of the two adenine atoms, N-1 or C-2, which under the influence of 3-AT pass to the medium within the IG molecule. If this were the case, 3-AT would affect the metabolism of the presumed precursor in two ways: by inhibiting its synthesis and by increasing the demand for it. In the presence of histidine, when 3-AT does not increase the demand for the precursor, the inhibition of its biosynthesis would be insufficient to make this process growth-limiting. At high 3-AT concentration the inhibition of the synthesis of the precursor could make it the growth-limiting process even in the presence of an excess of histidine in the cells.

On the basis of the presented results it appears that cell impermeability to 3-AT can be excluded as the mechanism rendering yeast resistant to this inhibitor. The existence of two 3-AT sensitive reactions in yeast suggests the possible occurrence of two different mechanisms of resistance development. The resistance acquired at low concentration of 3-AT or at high concentration of 3-AT in the presence of histidine, i.e. when the synthesis of adenine and cytosine is the growth-limiting factor, could be due to derepression of the sensitive enzyme catalysing the reaction required for the synthesis of adenine and cytosine. The resistance acquired at low concentration of 3-AT in the presence of adenine or cytosine, or at high concentration of 3-AT alone , i.e. when histidine biosynthesis becomes the growth-limiting factor, might result from a derepression of IGP dehydratase. The presented results are not in disaccord with this supposition but positive findings are required to support it.

REFERENCES

[1] Ames B. N. - *J. Biol. Chem.* **228**, 131, 1957.

[2] Ames B. N. & Hartman P. E. *The Molecular Basis of Neoplasia*, p. 322. University of Texas Press. Austin, 1962.

[3] Ames B. N. & Hartman P. E., *Cold Spring Harbour Symposia Quant. Biol.* **28**, 349, 1963.

[4] Bagdasarian G. & Kłopotowski T., *Abstracts 6th Int. Congress Biochem., New York*, 1964. p. 713.

[5] Fink G. R. - *Science* **146**, 525, 1964.

[6] Galzy P. & Slonimski P. P. - *C. R. Acad. Sci. Paris* **245**, 2423, 1957.

[7] Hilton J. L. - *Weeds* **8**, 392, 1960.

[8] Hilton J. L., Jansen L. L. & Hull H. M. - *Ann. Rev. Plant. Physiol.* **14**, 353, 1963.

[9] Hilton J. L. & Kearney P. C. - *Weeds* **13**, 22, 1965.

[10] Hilton J. L., Kearney P. C. & Ames B. N. - *Arch. Biochem. Biophys.* **112**, 544, 1965.

[11] Kłopotowski T. - *Acta Biochim. Polon.* **10**, 199, 1963.

[12] Kłopotowski T. & Hulanicka D. - *Acta Biochim. Polon.* **10**, 209, 1963.

[13] Kłopotowski T., Luzzati M. & Slonimski P. P. - *Biochem. Biophys. Res. Commun.* **3**, 150, 1960.

[14] Kłopotowski T. & Wiater A. - *Arch. Biochem. Biophys.* **112**, 562, 1965.

[15] Martin R. G. - *J. Biol. Chem.* **238**, 257, 1963.

[16] Shedlovsky A. E. & Magasanik B. - *J. Biol. Chem.* **237**, 3725, 1962.

[17] Weyter F. W. & Broquist H. P. - *Biochim. Biophys. Acta* **40**, 567, 1960.

CZĘŚCIOWE ZNOSZENIE PRZEZ ZASADY PURYNOWE I PIRYMIDYNOWE INHIBICJI WZROSTU DROŻDŻY POWODOWANEJ PRZEZ 3-AMINO-1,2,4-TRIAZOL

Streszczenie

1. Stwierdzono, że gdy 3-amino-1,2,4-triazol (3-AT) jest obecny w pożywce w niskich stężeniach, poniżej 2 mM, wzrost dzikiego szczepu *S. cerevisiae* jest ograniczony przez syntezę adeniny. Natomiast przy wysokim stężeniu 3-AT (20 mM) biosynteza histydyny staje się procesem limitującym wzrost drożdży.

2. Doświadczenie z użyciem mutanta hi_3 *S. cerevisiae* wykazało, że poza dehydratazą imidazologlicerolofosforanu, drożdże zawierają inny wrażliwy na 3-AT enzym niezbędny dla biosyntezy adeniny i cytozyny.

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THE EFFECT OF REPRESSION OF THE HISTIDINE OPERON ON THE SYNTHESIS OF RAPIDLY LABELLED RNA IN *S. TYPHIMURIUM*

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1. The repression of all amino acid synthesizing enzymes by the addition of casein hydrolysate leads to a marked drop in the rate of synthesis of the total rapidly labelled RNA. The addition of histidine alone does not produce this effect. 2. A 15 to 20% drop in the rate of labelling of the RNA after the addition of histidine is observed if the interference of radioactivity due to ribosomal RNA is eliminated from determinations by counting only the RNA complementary to homologous DNA.

Current views on the regulatory mechanisms of protein synthesis postulate that the repression and induction of enzyme synthesis is exerted at the level of gene transcription, i.e. the production of messenger RNA [9]. This theory has been confirmed by direct measurement of the amount of the specific messenger produced in *E. coli* in response to the inducer of β-galactosidase [6]. By using the double-labelling technique and cochromatography of RNA Martin [13] showed that *S. typhimurium* mutants constitutive for histidine synthesis produce more of the specific polycistronic messenger than the mutants carrying a deletion of the histidine operon. Hiatt *et al.* [7] have demonstrated the decrease in the overall labelling of RNA during the repression of all amino acid synthesizing enzymes in *E. coli*. In the present paper some experiments on the synthesis of the rapidly labelled RNA in *Salmonella typhimurium* during the repression of the histidine operon are presented.

MATERIALS AND METHODS

Strains and media. The following strains of *Salmonella typhimurium* were a kind gift of Dr. B. Ames: LT-2 the wild type, *his* G-70 a point mutant in the first gene of the histidine operon, which is the structural gene for phosphoribosylpyrophosphate - ATP pyrophosphorylase.

The strains were cultivated on mineral medium [3] supplemented with 0.5% glucose, and different concentrations of L-histidine or *N*-formylhistidine as indicated in the text. When large amounts of the repressed cells were required for the preparation of DNA the above medium was supplemented with 0.5% yeast extract (Difco) and 0.2% acid casein hydrolysate.

For the labelling experiments with [^{32}P]orthophosphate the tris minimal medium of Kjeldgaard [10] from which sodium phosphate was omitted, was used.

Analytical methods. Protein was determined by the method of Lowry *et al.* [11]. RNA was determined by a modification of the orcinol method of Mejbaum [Horecker *et al.*, 8]. The imidazoleglycerolphosphate dehydratase (EC 4.2.1.19) was assayed as described by Ames [1]. The activity was expressed in μmoles of imidazole acetol phosphate (IAP) formed per mg. protein per hr.

Special reagents. *Nα*-Formyl-L-histidine was synthesized by the method of Fischer [5], DNA was isolated by the method of Marmur [12], and RNA by the method of Scherrer & Darnell [15]. Imidazoleglycerolphosphate prepared by the method of Ames [1] was a kind gift of Dr. T. Kłopotowski. [2-^{14}C]Uracil was a product of Amersham Radiochemical Centre, England. $Na_2{}^{32}PO_4$ was obtained from the Institute for Nuclear Research, Warsaw. Acid casein hydrolysate was a product of Difco Lab. (Detroit, U.S.A.), L-histidine of Chemapol, Czechoslovakia, and crystalline ribonuclease of C. F. Boehringer and Soehne, Mannheim, Germany.

RESULTS AND DISCUSSION

The derepression of the enzymes of histidine biosynthesis was obtained by cultivating the mutant on limited amounts of exogenous histidine. This was achieved by substitution of *N*-formylhistidine for histidine in the medium as described by Ames & Garry [2]. The results of a typical experiment are presented in Fig. 1. Usually 15- to 25-fold derepression was obtained by this method.

The incorporation of RNA precursors into the rapidly labelled RNA was followed by the addition of [2-^{14}C]uracil at the final concentration of 0.17 μc/ml. (0.4

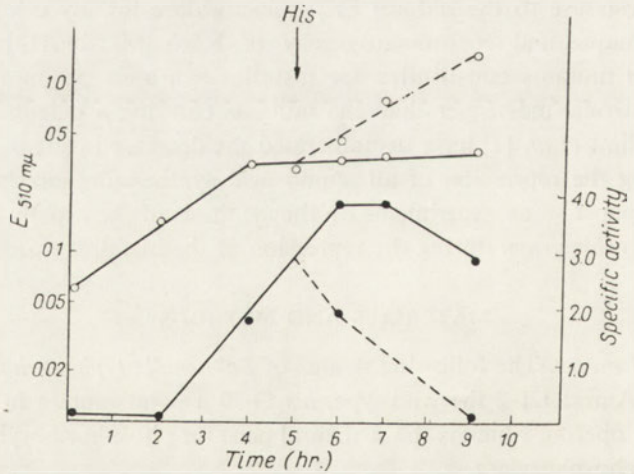

Fig. 1. The derepression and repression of the synthesis of imidazoleglycerolphosphate dehydratase in the point mutant *his* G-70 of *S. typhimurium*. Cells were cultivated at 37° with shaking on mineral medium with 0.5% glucose and 0.03 mM-*N*-formylhistidine. Portions of the culture were withdrawn for (○), extinction and (●), enzyme determination. At 5 hr. the culture was divided into two portions and (- - -), L-histidine was added to one of them to a final concentration of 0.02 mM.

μmoles/ml.) to the bacterial cultures containing 8×10^8 cells/ml. The cultures were aerated by shaking at 19°. One minute prior to the addition of the labelled compound, casein hydrolysate at the final concentration of 0.5% or L-histidine at final concentration of 0.02 mM were added to the culture in order to obtain the repression. Portions of the labelled culture were withdrawn at 1 min. intervals and pipetted into cold 10% trichloroacetic acid, and the precipitate washed 3 times with cold trichloroacetic acid, once with acetone and once with ether. The dry residues were dissolved in 0.2 N-KOH and counted in a liquid scintillation counter in a dioxan-based scintillator [4]. Crystalline ribonuclease added to the aqueous suspension of the above precipitates rendered 89% of the radioactivity acid-soluble which indicated that practically all the radioactivity in these precipitates belonged to the ribonucleic acids. Chasing experiments indicated that about 50% of the radioactivity could be chased out from the labelled cells within 3 min. by the addition of an excess of unlabelled uracil.

When the rate of labelling of the rapidly labelled RNA was studied in the derepressed culture of the point mutant *his* G-70, a decrease in the overall labelling of the RNA was observed when casein hydrolysate was added to this culture one minute before the start of the labelling, in accordance with the results obtained

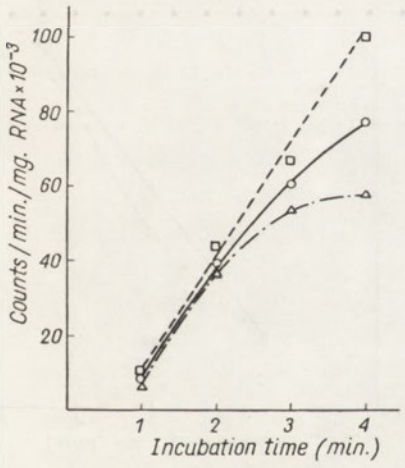

Fig. 2

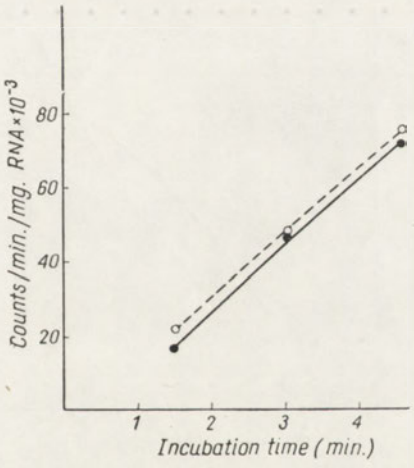

Fig. 3

Fig. 2. The effect of histidine and casein hydrolysate on the incorporation of [2-^{14}C]uracil into the total RNA of the derepressed culture of the point mutant *his* G-70 of *S. typhimurium*. (○), No additions; (△), casein hydrolysate added to a final concentration of 0.5%; (□), L-histidine added to a final concentration of 0.02 mM one min. before the start of the labelling.

Fig. 3. The effect of histidine on the incorporation of [^{32}P]orthophosphate into the total RNA of the derepressed culture of the *his* G-70 mutant of *S. typhimurium*. (●), No additions; (○), L-histidine added one min. before the start of the labelling to a final concentration of 0.02 mM.

by Hiatt *et al.* [7] with *E. coli*. When, however, we tried to obtain a similar effect by repressing only the histidine operon by the addition of histidine no such decrease was observed. On the contrary a slight increase in the rate of labelling of the RNA was usually obtained. These results are presented in Fig. 2.

The apparent negative result of the attempt to demonstrate the effect of the repression on the rate of labelling of the rapidly labelled RNA might be explained in the following way. The increase in the synthesis of the ribosomal RNA due to the increased growth rate on addition of histidine to the histidine-deprived cells is very rapid and therefore makes impossible the detection of the expected effect of the repression on the labelling of total RNA. If this supposition were correct it should be possible to detect the expected drop in the synthesis of the messenger RNA fraction provided that the radioactivity due to ribosomal RNA could be eliminated.

Elimination of this ribosomal RNA "noise" was attempted in the next experiment by studying only the radioactivity of the RNA complementary to the homologous DNA. It has been established by Yankofsky & Spiegelman [17] that only about 0.3% of the ribosomal RNA in bacteria is complementary to the homologous DNA. For the hybridization experiments the derepressed culture of the mutant *his* G-70 was centrifuged, washed and suspended in tris minimal medium at the density of about 10^9 cells/ml. $Na_2H^{32}PO_4$ at final concentration of 15 μc/ml. (5 μmoles/ml.) was then added to the control culture and to the culture to which

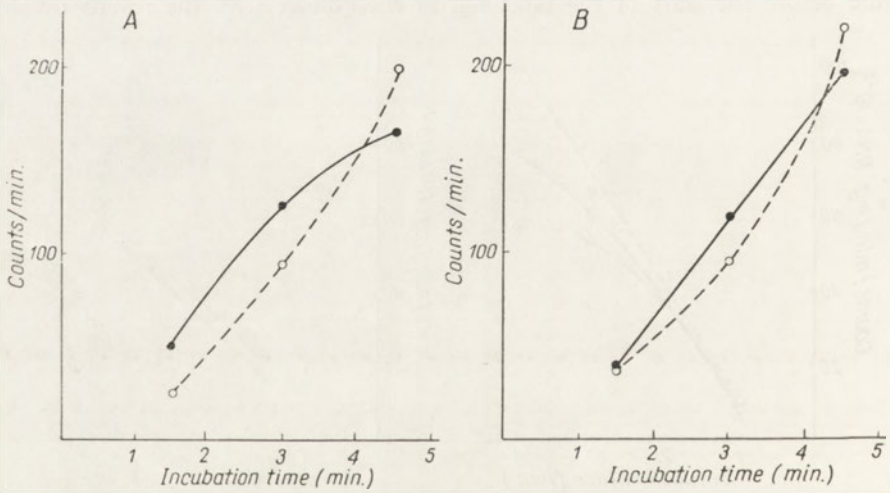

Fig. 4. The effect of histidine on the rate of the labelling of the RNA complementary to homologous DNA in *S. typhimurium his* G-70. *A*, Radioactivity of the RNA-DNA hybrids determined by the method of Nygaard & Hall [14]. *B*, Ribonuclease-resistant radioactivity in the DNA-RNA hybrids. (●), No additions; (○), L-histidine added one min. before the start of the labelling to a final concentration of 0.02 mM.

L-histidine was added at final concentration of 0.02 mM one minute before the start of the labelling, in the same way as described above for the incorporation of uracil. Portions of the cultures were withdrawn at 1.5 min. intervals, poured on the frozen tris minimal medium and used for the preparation of RNA.

When the specific activity of total RNA was determined after this experiment no significant difference was observed in the overall rate of the labelling between the derepressed and repressed cultures (Fig. 3).

Twenty μg. of the labelled RNA were hybridized with 45 μg. of heat-denatured DNA isolated from the wild type *S. typhimurium* LT-2, in 0.5 ml. of 0.01 M-tris buffer, pH 7.3, containing 0.5 M-KCl. The hybridization was carried out by heating the mixture for 3 hr. at 75° in an ultrathermostat containing 7 litres of water and then allowing the thermostat to cool slowly overnight to room temperature. The radioactivity of hybridized RNA was measured by adsorbing the hybrids on the nitrocellulose membrane filters as described by Nygaard & Hall [14]. The advantage was also taken of the well known property of the DNA-RNA hybrids: their resistance to ribonuclease digestion [16], and the ribonuclease-resistant radioactivity was determined in the hybridized mixtures. No attempts were made, however, to separate the hybrids by density gradient centrifugation or chromatography.

The results of the determination of the rate of synthesis of complementary RNA after the addition of histidine to the derepressed cultures are presented in Fig. 4. Both techniques used for the detection of the complementary RNA gave qualitatively the same results indicating that a transient drop in the rate of synthesis of complementary RNA occurs during the repression of the histidine operon. A 20% drop in the rate of the labelling of the complementary RNA after repression of the histidine operon appears unexpectedly high if it is remembered that the enzymes of histidine biosynthesis constitute only about 2% of the total protein in an eightfold derepressed constitutive mutant (cf. Martin [13]). It must be borne in mind, however, that the results of the labelling experiments described above give indications concerning the rate of the synthesis of complementary RNA but do not represent the differences in the amount of messenger formed.

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

REFERENCES

[1] Ames B. N. - *J. Biol. Chem.* **228**, 131, 1957.

[2] Ames B. N. & Garry B. - *Proc. Natl. Acad. Sci. U.S*, **45**, 1453, 1959.

[3] Ames B. N. & Garry B. - *J. Gen. Microbiol.* **22**, 369, 1960.

[4] Bray G. A. - *Anal. Biochem.* **1**, 279, 1960.

[5] Fischer E. & Cone L. H. - *Liebigs Annalen* **363**, 107, 1908.

[6] Hayashi M., Spiegelman S., Franklin N. C. & Luria S. E. - *Proc. Natl. Acad. Sci. U.S.* **49**, 729, 1963.

[7] Hiatt H. H., Gros F. & Jacob F. - *Biochim. Biophys. Acta* **72**, 15, 1963.

[8] Horecker B. L., Smyrniotis P. Z. & Klenow H. - *J. Biol. Chem.* **190**, 661, 1951.

[9] Jacob F. & Monod J. - *J. Mol. Biol.* **3**, 318, 1961.

[10] Kjeldgaard N. O. - *Biochim. Biophys. Acta* **49**, 64, 1961.

[11] Lowry O.H., Rosebrough N. J., Farr A. L. & Randall R. J. - *J. Biol. Chem.* **193**, 265 , 1951

[12] Marmur J. - *J. Mol. Biol.* **3**, 208, 1961.

[13] Martin R. G. - *Cold Spring Harbour Symp. Quant. Biol.* **28**, 357, 1963.

[14] Nygaard A. P. & Hall B. D. - *Biochem. Biophys. Res. Commun.* **12**, 98, 1963.
[15] Scherrer K. & Darnell J. E. - *Biochem. Biophys. Res. Commun.* **7**, 486, 1962.
[16] Yankofsky S. A. & Spiegelman S. - *Proc. Natl. Acad. Sci. U.S.* **48**, 1069, 1962.
[17] Yankofsky S. A. & Spiegelman S. - *Proc. Natl. Acad. Sci. U.S.* **49**, 538, 1963.

WPŁYW REPRESJI OPERONU HISTYDYNY NA SYNTEZĘ SZYBKO ZNAKUJĄCEGO SIĘ RNA U *S. TYPHIMURIUM*

Streszczenie

1. Represja wszystkich enzymów syntezy aminokwasów przez dodatek hydrolizatu kazeiny prowadzi do widocznego zmniejszenia szybkości syntezy całkowitego szybko znakującego się RNA. Efektu tego nie obserwuje się jednak po dodaniu tylko samej histydyny.

2. Zmniejszenie 15 do 20 procentowe szybkości syntezy RNA po dodaniu histydyny do dereprymowanych hodowli daje się zaobserwować jedynie wówczas, gdy z oznaczeń eliminuje się radioaktywność pochodzącą od rybosomalnego RNA poprzez oznaczanie aktywności właściwej tylko RNA komplementarnego do homologicznego DNA.

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SOME PROPERTIES OF HUMAN PLACENTAL SOLUBLE ARYLSULPHATASES

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1. Arylsulphatase A and B has been shown to occur in water extracts of acetone-dried powder from human placenta. 2. Dialysis against water separated both enzymes; arylsulphatase B precipitated with euglobulins, while arylsulphatase A remained in solution. 3. Arylsulphatase B activity in placenta washed by water perfusion was lower than in the unwashed tissue. The difference in arylsulphatase A activity was negligible. 4. Influence of various substances on the activity of the two enzymes separated by dialysis has been tested.

Human placenta contains most of the known enzymes [5] including arylsulphatases (arylsulphate sulphohydrolase EC 3.1.6.1). Although the physiological significance of arylsulphatases is not yet known, their wide distribution in mammalian tissues may point to an important role in general metabolism. Bianchi [2, 3] was the first to demonstrate arylsulphatase activity in human placenta with the use of *p*-nitrophenyl sulphate as substrate. The findings of Bianchi were confirmed by Pulkkinen [7]. Gniot & Działoszyński [6] found that human placenta contains soluble arylsulphatases (type II) which preferably hydrolyse 2-hydroxy-5-nitrophenyl sulphate and arylsulphatase C (type I) which has greater affinity to *p*-nitrophenyl sulphate. As it was shown by Pulkkinen [8] placenta exhibits also steroid sulphatase activity.

This paper, being a continuation of our previous work [6], describes a simple method for the separation of arylsulphatases A and B and the influence of various substances on the activity of both enzymes.

MATERIAL AND METHODS

Water extracts from acetone-dried powder prepared from fresh human placenta served as the source of enzymes. Each placenta was divided into two roughly equal parts. One part was washed by perfusing the blood vessels with distilled water, until the tissue became distinctly paler, the other was rinsed. The method of preparing acetone-dried powder and water extract was described in a previous paper

[6]. Enzyme extract was dialysed in visking tubing against distilled water at 4° to 10° for 50 - 60 hr.; 40 - 60 l. of water were used for 20 ml. of extract. The water was changed every 10 - 12 hr. Each dialysed extract was made up with water to twice its original volume. During dialysis a precipitate of euglobulins appeared, which was centrifuged at 13 000 *g* for 30 min. at 5°. After separation of the supernatant, the precipitate was washed with water and again centrifuged, the washings discarded and the precipitate from 20 ml. of the original extract was suspended in the same volume of water. Enzyme activity was measured in crude extract, in the whole extract after dialysis, in the supernatant and in the suspension of the precipitate.

Estimation of enzyme activity. The activity of arylsulphatases A and B was estimated as described before [6], with dipotassium 2-hydroxy-5-nitrophenyl sulphate as substrate in concentration of 3 mM and 43.6 mM in 0.5 M-acetate buffer, pH 4 - 7, at 37°. Incubation time 1 hr. In experiments on the influence of various substances on enzyme activity the actual concentration of the tested substance was 0.1 or 0.001 M. Substrate concentration in this case was 3 mM and 10 mM for arylsulphatases A and B, respectively.

RESULTS AND DISCUSSION

According to the findings of other workers, arylsulphatase A exhibits maximum activity at pH 5.1 [11] or 4.4 and 5.0 [1]. This enzyme shows greater affinity to 2--hydroxy-5-nitrophenyl sulphate [9] than arylsulphatase B [10] which acts optimally at pH 6.1 or 6.2 [4, 11].

Having this in mind we determined the optimum pH values for the enzymic extract from placenta, using two concentrations of substrate: 3 mM which is the optimum for arylsulphatase A and 43.6 mM, nearly optimum for arylsulphatase B. At 3 mM-2-hydroxy-5-nitrophenyl sulphate concentration the dialysed as well as most of the non-dialysed extracts showed only one pH optimum. At 43.6 mM substrate two optima appeared in dialysed and non-dialysed extracts corresponding roughly to the optima of arylsulphatases A and B (Fig. 1a).

In non-dialysed extract, enzyme activity was higher at 43.6 mM than at 3 mM substrate concentration, while after dialysis the activity at 3 mM exceeded that at 43.6 mM (Fig. 1a, b). Dialysis apparently removed substances acting as competitive inhibitors. Analysis of the dialysed extract pointed to phosphate as being this inhibitor. Addition of phosphate in an amount corresponding to that found in the extract, reversed the picture making the relationship between enzyme activity and pH similar to that existing in the non-dialysed extract.

Table 1 shows the mean optimum pH values for dialysed and non-dialysed extracts from six placentas. It may be seen that at low substrate concentration (3 mM), dialysis caused a general shift of the optimum pH towards the acid side. The shift was much smaller at higher (43.6 mM) concentration of the substrate. This is in agreement with the results of Roy [11], who showed that phosphate causes a shift of the optimum pH of arylsulphatase towards the alkaline side especially at low

substrate concentration (arylsulphatase A). The shift observed by us, was caused most probably by the removal of phosphate.

Figure 1c shows that the supernatant which remained after separation of the precipitate appearing during dialysis, contained mostly arylsulphatase A with an optimum pH at 4.8. The suspension (Fig. 1d) of the precipitate, on the other hand,

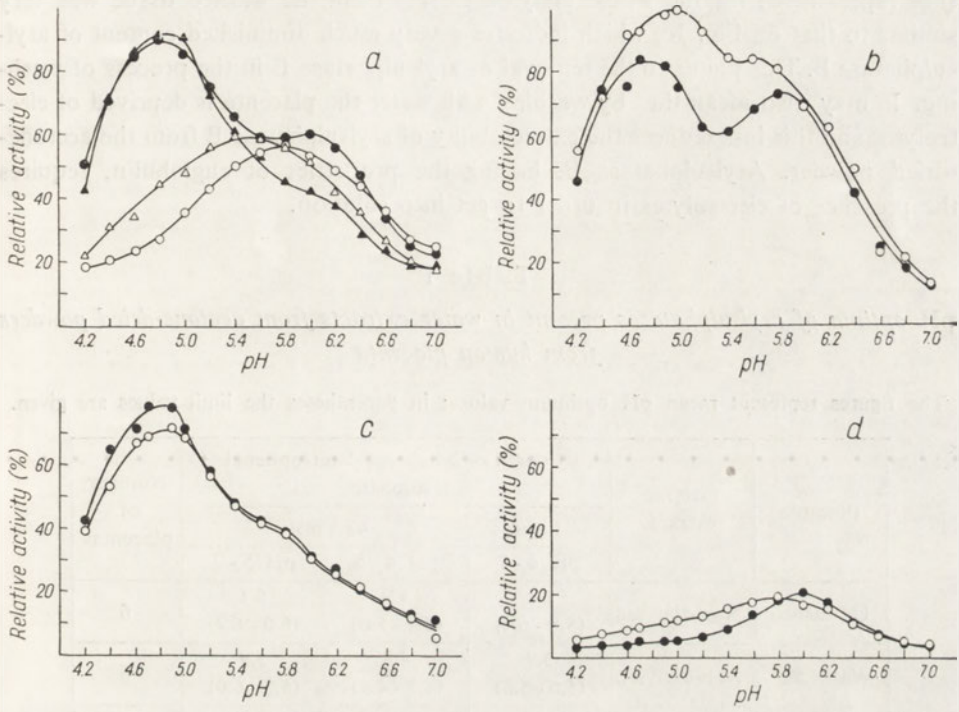

Fig. 1. Effect of pH on the activity of soluble arylsulphatases in water extracts of acetone-dried powders from human placentas. The activity represented by all curves is comparable with respect to enzyme concentration. The maximum activity: 20.2 μm-moles of 4-nitrocatechol/min./0.2 ml. of enzyme extract, was taken as 100. (○) or (△), at 3 mM concn. of 2-hydroxy-5-nitrophenyl sulphate; (●) or (▲), at 43.6 mM concn. *a*, Non-dialysed extract: (○, ●), from unwashed placenta; (△, ▲), from washed placenta. *b*, Dialysed extract from unwashed placenta. *c*, Dialysed extract from unwashed placenta after separation of euglobulins. *d*, Suspension of euglobulins.

was composed mainly of arylsulphatase B, with an optimum at pH 6.0. These characteristic pH optima appeared at low as well as at high concentration of the substrate. This means that a satisfactory separation of those two enzymes by a simple technique of dialysis can be achieved.

Washing of placenta through blood vessels with water reduced the activity of arylsulphatase B. As may be seen on Fig. 1a, which represents the results of a typical experiment, washing of placenta caused a drop of arylsulphatase B activity from 12.3 μm-moles of nitrocatechol/min. in the unwashed preparation to 8.75 in the washed one at 43.6 mM substrate concentration. The drop of activity of arylsulphatase A resulting from washing of the placenta was negligible.

There are also marked shifts of optimum pH from the washed to the unwashed preparation at low substrate concentration (Table 1). These shifts were from pH 6.1 to 5.7 in non-dialysed preparations and from pH 5.5 to 4.6 in the dialysed ones. This phenomenon was less distinct in non-dialysed extracts which contained phosphate shifting the optimum pH towards the alkaline side. The pH activity curve (not represented) for the whole dialysed extract from the washed tissue was very similar to that on Fig. 1c, which indicates a very much diminished content of arylsulphatase B. This points to the removal of arylsulphatase B in the process of washing. It may also mean that by washing with water the placenta is deprived of electrolytes and this loss reduces the extractability of arylsulphatase B from the acetone-dried powder. Arylsulphatase B, having the properties of euglobulin, requires the presence of electrolytes in order to get into solution.

Table 1

pH *optima of arylsulphatases present in water extracts from acetone-dried powders from human placenta*

The figures represent mean pH optimum values; in parentheses the limit values are given.

Placenta	Enzyme extract	Concn. of 2-hydroxy-5-nitrophenyl sulphate			Number of placentas
		3 mM	43.6 mM		
		pH 4 - 7	pH 4 - 5	pH 5 - 7	
Unwashed	Non-dialysed	6.1 (5.8 - 6.4)	4.9 (4.7 - 5.0)	6.1 (6.0 - 6.2)	6
Washed	Non-dialysed	5.7 (5.6 - 5.8)	4.8 (4.7 - 4.9)	5.9 (5.8 - 6.0)	6
Unwashed	Dialysed	5.5 (5.0 - 5.8)	4.85 (4.7 - 4.9)	5.8 (5.8 - 5.9)	4
Washed	Dialysed	4.6 (4.6 - 4.8)	4.7 (4.6 - 4.8)	5.6 (5.6 - 5.8)	5

The concentration of inorganic phosphate in 1 ml. of enzyme extract from unwashed placenta was 1.33 mg. P and in that from the washed tissue it was 0.71 mg.

Results obtained in the previous work [6] support the view that by the procedure of washing part of arylsulphatase B was removed from the placenta. Those results showed that the residue of extract from acetone-dried washed placenta did not contain more arylsulphatase B, as might have been expected if by washing only electrolytes were removed.

Table 2 presents the results of a study on the influence of various substances on the activity of placental arylsulphatases A and B separated by dialysis. The study was undertaken with the aim of finding such substance which would inhibit one enzyme and be without effect on the other. Among the compounds examined the following cations at 1 mM concentration seemed to have no influence on the activity of either arylsulphatase: Ba^{2+}, Cd^{2+}, Mg^{2+}, Ni^{2+}, Sn^{2+}. The cations Co^{2+},

Mn^{2+} and Ce^{2+} caused slight activation. Zn^{2+}, Cu^{2+}, Al^{3+} and Fe^{3+} inhibited both arylsulphatases. Ag^{+} inhibited very markedly arylsulphatase A (about 66%) and to a lesser extent arysulphatase B (26%).

Table 2

Influence of various substances on placental arylsulphatases A and B separated by dialysis

Arylsulphatase activity was assayed using 2-hydroxy-5-nitrophenyl sulphate as substrate. The results are expressed as percentages of the control value.

Substance (1 mM concn.)	Arylsulphatase		Substance (0.1 M concn.)	Arylsulphatase	
	A	B		A	B
$Mg(CH_3COO)_2$	102.46	103.11	NaCl	97.99	60.54
$AlCl_3$	91.98	78.84	KCl	99.97	50.09
$CaCl_2$	107.13	98.98	NaF	10.75	13.08
$Mn(CH_3COO)_2$	110.00	106.88	NaN_3	104.14	43.15
$FeCl_3$	90.67	97.77	Na_2SO_4	26.87	55.83
$Co(CH_3COO)_2$	106.85	108.11	Na_2SO_3	1.62	4.65
$Ni(CH_3COO)_2$	98.90	100.11	KH_2PO_4	2.61	4.93
$CuCl_2$	83.21	83.21	$CH_2BrCOOH$	24.04	12.34
$Zn(CH_3COO)_2$	93.73	92.60	Sodium citrate	21.39	20.52
$Ag(CH_3COO)$	34.43	73.69	Sodium versenate (0.01 M concn.)	105.58	94.84
$CdCl_2$	101.31	101.32			
$SnCl_2$	98.69	102.11			
$Ba(CH_3COO)_2$	101.05	100.34			
$Ce(CH_3COO)_2$	101.65	107.92			

The anionic compounds exerted an inhibitory influence on both enzymes, the degree of inhibition depending on the particular substance. Sulphite, phosphate and fluoride are the chief inhibitors of both arylsulphatases. Special attention must be paid to sodium chloride and sodium azide which have no effect on arylsulphatase A and quite strongly inhibit arylsulphatase B. Also by applying such inhibitory ions as SO_4^{2-} and Ag^{+} and $CH_2BrCOOH$ the activities of both enzymes can be to some degree differentiated.

These observations might be useful for working out a method for the estimation of either enzyme in a mixture.

REFERENCES

[1] Baum H., Dodgson K. S. & Spencer B. - *Biochem. J.* **69**, 567, 1958.
[2] Bianchi M. - *Pathologica (Genova)* **47**, 39, 1955.
[3] Bianchi M. & Valli P. - *Chem. Abstr.* **50**, 3598a, 1956.
[4] Dodgson K. S. & Wynn C. H. - *Biochem. J.* **68**, 387, 1958.
[5] Flexner L. B., *Gestation, Transaction of the first Conference, March* 9, 10 *and* 11, *Josiah Macy Jr. Foundation, New York*, 1954.
[6] Gniot J. & Działoszyński L. M. - *Clin. Chim. Acta* **9**, 334, 1964.
[7] Pulkkinen M. O. - *Acta Physiol. Scand.*, Suppl. 145, **42**, 125, 1957.
[8] Pulkkinen M. O. - *Acta Physiol. Scand.*, Suppl. 180, **52**, 1, 1961.

[9] Roy A. B. - *Biochem. J.* **55**, 653, 1953.
[10] Roy A. B. - *Biochem. J.* **57**, 465, 1954.
[11] Roy A. B. - *Biochem. J.* **59**, 8, 1955.

NIEKTÓRE WŁASNOŚCI ROZPUSZCZALNYCH ARYLOSULFATAZ ŁOŻYSKA LUDZKIEGO

Streszczenie

1. Ekstrakty wodne z acetonowych preparatów tkanki łożyska ludzkiego zawierają dwie rozpuszczalne arylosulfatazy A i B.

2. Enzymy ulegają rozdzieleniu w trakcie dializy. Arylosulfataza B wytrąca się z osadem euglobulin, natomiast arylosulfataza A pozostaje w roztworze.

3. Z porównania aktywności enzymów A i B w ekstraktach z łożysk ukrwionych i po usunięciu krwi wynika, że te ostatnie zawierają znacznie mniej arylosulfatazy B.

4. Przebadano wpływ kilkunastu związków na aktywność arylosulfataz A i B rozdzielonych w trakcie dializy.

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PREPARATION AND PROPERTIES OF SOME METHYL-SUBSTITUTED CYTOSINE RIBOSIDES AND THEIR INTERMEDIATES, AND POLY-5--METHYLCYTIDYLIC ACID

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1. Syntheses and properties are described for a number of exocyclic monomethylamino and dimethylamino analogues of cytosine, 1-methylcytosine and cytidine, and cytidine-5′--phosphate and cytidine-5′-pyrophosphate. The methylamino analogues of the latter proved to be substrates of polynucleotide phosphorylase. 2. The mechanism of the condensation reaction leading to the formation of the nucleosides was examined in some detail. 3. The p*K* values and ultraviolet absorption spectra of the different ionic forms of the various derivatives are also presented and discussed. 4. The 5′-mono- and pyro-phosphates of 5-methylcytidine have also been synthesized; the latter of these was likewise found to undergo polymerization by polynucleotide phosphorylase.

It was shown in previous communications that methylation of the ring $N_{(3)}$ nitrogens in poly-U and poly-5MeU (or poly-rT)[1] led to a complete loss in secondary structure of these polymers, as well as their ability to form multistranded complexes with poly-A, in agreement with expectations [29, 33]. In marked contrast to this was the observation that introduction of a 5-methyl substituent in poly-U (to give poly-5MeU) resulted in formation of a polymer with a markedly enhanced secondary structure and stability, and which formed with poly-A a twin-stranded complex the T_m of which was about 20° higher than that for poly-(A+U). It was subsequently further shown that poly-(I+5MeC) was also considerably more stable than poly-(I+C), the former exhibiting a T_m about 18° above that for the latter [30, 35].

As part of the above programme, attempts were at one time undertaken to prepare analogues of poly-C in which one or both of the exocyclic amino hydrogens were replaced by methyl groups. This work was subsequently abandoned

[1] The following abbreviations are used in this text: poly-U, poly-uridylic acid; poly-5MeU (or poly-rT), poly-ribothymidylic acid; poly-A, poly-adenylic acid; poly-C, poly-cytidylic acid; poly-5MeC, poly-5-methylcytidylic acid; poly-(A+U), twin-stranded complex of poly-A and poly-U, with similar connotations for other twin-stranded polymers.

when it was found that Brimacombe, Griffin & Reese (in press; Reese, personal communication) had succeeded in preparing such polymers. However, our procedures for the preparation of the necessary substrates and intermediates differed from those utilized by the foregoing authors.

The present communication describes the preparation and properties of the above-mentioned derivatives of cytosine and its nucleosides and nucleotides containing methyl substituents in the exocyclic amino group, or in the 5-positions of the pyrimidine ring, the various intermediates involved in these syntheses, and poly-5MeC (see Table 1). A number of these compounds have not hitherto been described in the literature. Some of them have already proven extremely useful in studies on the nature and properties of the photoproducts of cytosine and its glycosides [9] as well as in investigations on the mechanism of action of the mutagenic agent hydroxylamine [18, 19]. It is expected, in particular, that the spectra and other properties of the alkylamino cytosines and their glycosides will prove useful in the search for these constituents as "minor" bases in nucleic acids, particularly in s-RNA. Some of the phosphorylated analogues should also be of interest in studies on the substrate specificity of enzymes involved in nucleic acid metabolism (see below).

RESULTS AND DISCUSSION

5-Methylcytosine ribosides and poly-5MeC

5-Methylcytidine was obtained by thiation of 5-methyluridine and ammonolysis of the resulting 4-thio derivative as described by Fox *et al.* [12]. This was converted to 2′,3′-*O*-benzylidene-5-methylcytidine (XII) as described by Gulland & Smith [14] in 100% yield and the product obtained in crystalline form from

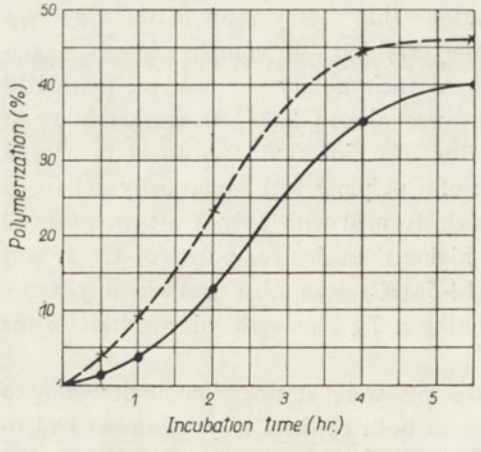


Fig. 1. Course of polymerization of (×), CDP and (●), 5-MeCDP with *Azotobacter vinelandii* polynucleotide phosphorylase.

aqueous ethanol. The benzylidene derivative was then phosphorylated with cyanoethylphosphate according to Tener [36] to yield 5-methylcytidine-5′-phosphate (XIII) in 68% yield. Conversion of the latter to the morpholine derivative, followed by phosphorylation as described by Moffat & Khorana [25] for cytidine-5′-pyrophosphate, gave 5-methylcytidine-5′-pyrophosphate (XIV) in essentially quantitative yield.

Table 1

R_F values of cytosine analogues and derivatives

Ascending chromatography, using Whatman no. 1 paper in following solvent systems: *A*, water-saturated butanol; *B*, the upper phase of benzene - ethanol - water (169 : 45 : 15, by vol.); *C*, propan-2-ol - water - ammonia (d, 0.88) (7 : 2 : 1, by vol.); *D*, ethanol - 1 M-sodium acetate (5 : 2, v/v); *E*, butanol, sat. with sat. solution of boric acid.

Compound	R_F in solvent				
	A	*B*	*C*	*D*	*E*
4-Ethoxyuracil	0.13*	0.10	—	—	—
1-Methyl-4-ethoxyuracil	0.80*	0.89	—	—	—
Cytosine	0.24*	0.02	—	—	—
1-Methylcytosine	0.34*	0.06	—	—	—
Monomethylaminocytosine (I)	0.45*	0.06	—	—	—
1-Methylmonomethylaminocytosine (III)	0.58*	0.13	—	—	—
Dimethylaminocytosine (II)	0.48*	0.15	—	—	—
1-Methyldimethylaminocytosine (IV)	0.63*	0.46	—	—	—
Product of condensation (V)	0.86	—	0.95	—	—
Cytidine	0.14	—	0.58	—	0.12
Monomethylaminocytidine (VI)	0.24	—	0.68	—	0.22
Dimethylaminocytidine (VII)	0.26	—	0.71	—	0.23
2′,3′-*O*-Isopropylidenemonomethylaminocytidine (VIII)	0.68	—	0.88	—	0.77
2′,3′-*O*-Isopropylidenedimethylaminocytidine (IX)	0.72	—	0.91	—	0.81
2′,3′-*O*-Benzylidenemonomethylaminocytidine (X)	0.14	—	—	—	0.83
2′,3′-*O*-Benzylidenedimethylaminocytidine (XI)	0.80	—	—	—	0.88
2′,3′-*O*-Benzylidene-5-methylcytidine (XVI)	0.71	—	—	—	0.80
Methylaminocytidine-5′-phosphate (XII)	—	—	0.18	0.26	—
Dimethylaminocytidine-5′-phosphate (XIV)	—	—	0.22	0.29	—
5-Methylcytidine-5′-phosphate (XVII)	—	—	0.16	0.25	—
Methylaminocytidine-5′-pyrophosphate (XIII)	—	—	—	0.06	—
Dimethylaminocytidine-5′-pyrophosphate (XV)	—	—	—	0.10	—
5-Methylcytidine-5′-pyrophosphate (XVIII)	—	—	—	0.06	—

* With ammonia in the vapour phase.

5-Methylcytidine-5′-pyrophosphate was polymerized with *Azotobacter vinelandii* polynucleotide phosphorylase under standard conditions [1] and the resulting polymer isolated as elsewhere described [31]. The course of polymerization of 5-MeCDP was followed by paper chromatography and is exhibited in Fig. 1. For

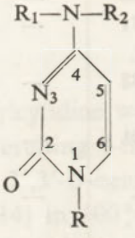
comparison purposes, a control run is demonstrated also for the course of polymerization of CDP. It will be noted that the behaviour of both substrates is very similar, both as regards rate of polymerization and amount of polymer formed at equilibrium.

By contrast poly-C was found to be hydrolysed by pancreatic ribonuclease at a rate about threefold that for poly-5MeC. This is due largely to the influence of the 5-methyl substituent since, as shown elsewhere [30], poly-C and poly-5MeC are structurally similar at the pH 7.2 employed for enzymic hydrolysis. This was further confirmed by the finding that cytidine-2′:3′-cyclic phosphate was hydrolysed by ribonuclease at a rate fourfold greater than that for 5-methylcytidine-2′:3′-cyclic phosphate.

Alkylamino cytosines and their intermediates

Pyrimidine syntheses. The pyrimidine analogues were prepared because it was felt that their spectral and other properties would prove useful in studies on the glycosides.

Mono- and di-methylaminocytosine and their 1-methyl analogues (compounds I, II, III and IV, respectively):



I : R = H; R_1 = H; R_2 = CH_3
II : R = H; R_1 = CH_3; R_2 = CH_3
III : R = CH_3; R_1 = H; R_2 = CH_3
IV : R = CH_3; R_1 = CH_3; R_2 = CH_3

were obtained by the action of methanolic solutions of mono- and di-methylamine, respectively, on 2-keto-4-ethoxypyrimidine [15] and 1-methyl-2-keto-4-ethoxypyrimidine [16]. The reactions were conducted in sealed tubes at 50° for 18 hr. to give, in all instances, essentially quantitative yields. The spectral properties of these compounds are discussed separately below in connection with those for the corresponding glycosides. They were all resistant to deamination in 1 N-KOH at 60° for 18 hr.

Riboside syntheses. Several pathways are available for the preparation of monomethylaminocytidine (VI) and dimethylaminocytidine (VII). These include: (i) the Hilbert-Johnson method; (ii) the "mercury" method; (iii) the "thiation" procedure, leading to appropriate amination of 4-thiouridine (all of these are reviewed in ref. [13]). The procedure finally adopted and described below was selected in part because it was hoped that the necessary starting compounds might prove useful in the preparation of 3-β-nucleosides, and partly because of the unavailability at that time of a sufficient quantity of uridine as starting product for the thiation procedure.

It is necessary to draw attention to the fact that, at the time these syntheses were carried out, the β-configuration of nucleosides obtained by the Hilbert-Johnson

or mercury methods was considered as generally established [13, 23]. However, it was subsequently shown that condensation of 2,4-dimethoxy-5-methylpyrimidine with tri-*O*-acetyl-D-ribofuranosyl bromide leads to formation of a mixture of both α- and β-anomeric nucleosides [8]. Furthermore, it should be recalled that the formation of anomers is quite common in the mercury method for pyrimidine deoxynucleosides [8]. It must be concluded that independent confirmation for formation of one or both anomers is now a prerequisite in all such condensation procedures, and this point must be borne in mind in what follows.

Attempts were made initially to obtain VI and VII by the mercury procedure *via* 1-β-(2-keto-4-ethoxy)-2′,3′,5′-tribenzoylribofuranosylpyrimidine (V), followed by methylamination and simultaneous debenzoylation of the condensation product. For this purpose trials were made to condense the mercury derivative of 2-keto-4--ethoxypyrimidine with 1-chloro-2,3,5-tribenzoylribofuranose [26]. This, however, gave, in addition to the required product V, 3-β-(2,4-diketo)-2′,3′,5′-tribenzoylribofuranosylpyrimidine [27]. Both glycosides, as well as their debenzoylated (by means of $NaOCH_3$) derivatives, V and the 3-β-uridine, were identified by paper chromatography and ultraviolet absorption spectra.

The foregoing result differs from that of Scannell & Allen [27], who reported that condensation of the Hg salt of 2-keto-4-ethoxypyrimidine with 1-chloro-2,3,5--tribenzoylribofuranose gave exclusively the 3-β-nucleoside. On the other hand, trial condensations according to the method of Hilbert & Johnson [17] gave only one product, i.e. V, with a reproducible yield of 40%. The starting compound for the condensation in this case was 2,4-diethoxypyrimidine, whereas the mercury procedure requires the less accessible 2-keto-4-ethoxypyrimidine. The only published procedure for the preparation of 2-keto-4-ethoxypyrimidine is based on the mild alkaline hydrolysis of 2,4-diethoxypyrimidine, in about 34% yield [15]. The other products of hydrolysis include 2-ethoxy-4-hydroxypyrimidine and uracil.

Several remarks are in order with respect to the condensation reactions. In our hands, as mentioned above, apparently only the classical Hilbert-Johnson condensation, where the possibilities of tautomerization of the systems $-\overset{\overset{H}{|}}{N}_{(1)}-\overset{\overset{O}{\|}}{C}_{(2)}$ and $-\overset{\overset{H}{|}}{N}_{(3)}-\overset{\overset{O}{\|}}{C}_{(4)}$ are excluded, gives only one product, the $N_{(1)}$ glycoside. This once more substantiates the general contention as to the greater basicity of the $N_{(1)}$ of the pyrimidine ring, as compared to $N_{(3)}$, and its consequent greater susceptibility to reaction with the halogeno sugar. On the other hand the use of 2-keto--4-ethoxypyrimidine, either in the free form or as the mercury salt, resulted in formation of both $N_{(1)}$ and $N_{(3)}$ glycosides. It is necessary, in this connection, to recall the work of Brown [5] on the possibility of migration of methyl groups in the pyrimidine ring *via* ring opening. Related to this is our observation on the susceptibility to acid of 2-keto-4-ethoxypyrimidine. During the preparation of this latter, it was found that on contact with the ion-exchanger Amberlite IR-120 (H^+) it underwent hydrolysis to uracil and simultaneously partially isomerized to the 2-etho-

xy and/or 3-ethyl derivative. Apparently a similar process occurs under the conditions of the mercury condensation procedure, which requires a relatively high temperature (about 140°, boiling point of xylene), and in the presence of the chloro-benzoylated sugar which appears to be rather labile under these conditions. This process might lead to the production of V under the reaction conditions described by Scannell & Allen [27] and is all the more probable in that, as the reaction time was increased, the yield of the $N_{(1)}$ glycoside increased with respect to the $N_{(3)}$. On the other hand, under the conditions described by Fox *et al.* [10], complexing with mercury does not appear to fully liquidate the possibility of tautomerization of the system $-\overset{H}{\underset{|}{N}}_{(1)}-\overset{O}{\underset{\|}{C}}_{(2)}-$ (as in 2,4-diethoxypyrimidine) and the structure of the pyrimidine component of the mercury complex favours glycosidation at $N_{(3)}$. Hence the presence of the 3-β-nucleoside in the reaction described by Fox *et al.* [10]. On the basis of the foregoing it appeared to us more profitable to apply the Hilbert-Johnson procedure to obtain VI and VII on a preparative scale.

The condensation of V was allowed to proceed for 48 hr. at an initial temperature of 65° which was raised at 12-hour intervals by 10° to a final temperature of 100°. In some experiments pure V was isolated and subjected to methylamination. The quantitative isolation of pure V proved, however, to be quite troublesome and was not essential; it sufficed to remove unreacted halogeno sugar and diethoxypyrimidine and subject the residual, oily, product to methylamination. The latter reaction was carried out in sealed tubes, in the presence of an excess of mono- or di-methylamine, initially at room temperature for 48 hr. [24] and finally at 60° for 12 hr. The amination reactions proceeded essentially quantitatively, both VI and VII crystallizing partially on concentration of the methanolic solutions and removal of excess amine. They were isolated quantitatively by adsorption on Dowex 50 (H^+) and elution with dilute HCl.

Isopropylidene derivatives (*VIII and IX*). The 2′:3′-*O*-isopropylidene derivatives of mono- and di-methylaminocytidine were obtained by treatment of the nucleosides with *p*-toluenosulphonic acid as described by Chambers *et al.* [7] for cytidine. The reactions did not go as smoothly, nor with such high yields, as with cytidine. This was tested by preparing isopropylidenecytidine under the same conditions, according to the published procedure [7], which indeed gave a practically quantitative yield. Only by increasing the reaction temperature to 37° and lengthening the reaction time from 2 to 24 hr. was the yield of VIII raised to 65% and that of IX to 56%. By contrast to 2′:3′-*O*-isopropylidenecytidine, which has been isolated only as a non-crystalline glass [7], it was found possible to obtain both VIII and IX in crystalline form.

Benzylidene derivatives (*X and XI*). The 2′,3′-*O*-benzylidene derivatives of VI and VII were prepared according to Gulland & Smith [14] by treatment of the nucleosides with benzaldehyde in the presence of anhydrous $ZnCl_2$. The yields in both instances were practically quantitative, as demonstrated by paper chromatography and the periodate reaction. However, the products could not be crystallized and were isolated as glasses, which were freeze-dried from aqueous ethanol.

5'-Monophosphates of VI and VII: The 5'-phosphate of methylaminocytidine was prepared from the isopropylidene (VIII) and benzylidene (X) derivatives by the "polyphosphate" [22] and β-cyanoethylphosphate [36] methods. The latter proved the method of choice with an overall phosphorylation yield of about 70%.

The cyanoethylphosphate reagent was employed for phosphorylation of unprotected dimethylaminocytidine, the reaction time being shortened in order to minimize phosphorylation of the 2'- and 3'-hydroxyls. Initial trials demonstrated that the optimum phosphorylation time at room temperature was about 8 hr. when the molar ratio of nucleoside to phosphorylating agent was 1 : 3. Under these conditions the final reaction mixture consisted of 5% unreacted nucleoside, 29% of the 5'-monophosphate, 10% of the 2'- and 3'-phosphates, and the remainder 2',5'- and 3',5'-diphosphates. The composition of the reaction mixture was determined by separation on a Dowex 1 X8 (Cl^-) column.

Ring $N_{(3)}$ methylation of cytosine glycosides. No reaction was observed on exposure of cytidine in dimethylformamide to diazomethane for periods up to 72 hr. This is in agreement with the earlier observations of Bredereck *et al.* [2] and Kenner *et al.* [20]. It is consequently all the more surprising that cytidine-5'-phosphate was found to be partially susceptible to this methylating agent (referring, of course, to the pyrimidine and ribose moieties and not to the phosphate hydroxyls which are readily esterified). The initial products were the mono- and di-methyl esters of cytidine-5'-phosphate; with a further excess of CH_2N_2 there resulted about 20% *N*-methylation. These products were obtained in initial experiments and identified by means of paper chromatography in several solvent systems, dependence of ultraviolet absorption spectrum on pH, periodate oxidation and ability to form borate complexes.

Exhaustive treatment of cytidine-5'-phosphate with CH_2N_2 in dimethylformamide gave the following products: (a), dimethyl ester, 30%; (b), *N*-methylated dimethyl ester, 20%; (c), $O_{(2')}$-methyl (or possibly $O_{(3')}$-methyl, cf. [34]) dimethyl ester, 35%; (d), unidentified product, 15%. The last product is methylated on the 2' (or 3') of the ribose and extensively methylated on the ring $N_{(3)}$ nitrogen as inferred from the absorption spectrum, λ_{max} 255 mμ at neutral pH, and its lack of variation with pH.

The influence of the phosphate group on the susceptibility to etherification by diazomethane of the *cis*-glycol hydroxyl(s) had been previously observed in the case of uridine-5'-phosphate [34], but in this instance we see that the 5'-phosphate residue also modifies the susceptibility of the ring $N_{(3)}$ nitrogen to undergo methylation.

$N_{(3)}$-Methylcytidine was obtained by treatment with diazomethane of fully acetylated cytidine as described by Kenner, Reese & Todd [20] for cytosine and 1-methylcytosine (10 moles acetic anhydride per mole cytidine in pyridine solution for 60 min. at 100°). Acetylation gave $O_{(2'),(3'),(5')}$-$N_{(4)}$-tetraacetylcytidine, with a characteristic ultraviolet absorption maximum at 245 mμ, and which was precipitated by removal of excess acetic anhydride and concentration of the pyridine solution. The crystalline derivative underwent quantitative methylation on $N_{(3)}$ by treatment with diazomethane in ethanol solution. The $N_{(3)}$-methyltetraacetylcyti-

dine was precipitated by addition of an excess of ether, dissolved in methanol and deacetylated catalytically with $KOCH_3$. The course of the reaction was easily followed by paper chromatography in water-saturated butanol, the R_F values being: cytidine 0.12; following acetylation, 0.73; following methylation, 0.82; following deacetylation, 0.18. The $N_{(3)}$-methylcytidine was extremely hygroscopic and was transformed to *N*-methyluridine in alkali, in agreement with Brookes & Lawley [3]. Its absorption spectrum is unaltered over the pH range 2 - 7, λ_{max} 277.5 mμ, λ_{min} 245 mμ. At pH 12, λ_{max} 265 mμ, λ_{min} 247 mμ; apparent p*K*, measured spectrally, ~8.9[2].

Several trials were also made to obtain $N_{(3)}$-methylcytidylic acid, the 2′-hydroxyl being protected, and subsequent hydrolysis facilitated, by using as starting compound cytidine-2′:3′-cyclic phosphate (cf. analogous procedure for *N*-methyluridylic acid [32]). This was fully acetylated (cf. [24]) and then exhaustively treated with diazomethane, which gave $N_{(3)}$ methylation quantitatively. However, during the subsequent operations (deacetylation, hydrolysis of the monoester group, and column chromatography) the compound was completely deaminated to *N*-methyluridylic acid. In view of this marked lability, further work in this direction was discontinued.

Ultraviolet absorption spectra and apparent pK *values for alkylamino cytosine analogues.* The absorption spectra of the alkylamino cytosines, 1-methylcytosines and cytidines are presented in Figs. 2, 3 and 4. The spectra were in all instances recorded over the pH range 1 to 14, but, for purposes of simplicity, only the neutral and ionic forms are shown in the figures. The intermediate curves at various pH values were employed for calculations of the *apparent* p*K* values of the various analogues at room temperature, 20°; these results, along with those of other observers, are tabulated in Table 2.

From Fig. 2a it will be seen that the spectra of the neutral, cationic and anionic forms of methylaminocytosine are essentially similar to those for cytosine itself [28]. By comparison with cytosine, the neutral form of methylaminocytosine possesses a much more pronounced inflexion at about 235 mμ. This latter is, in turn, almost completely obliterated by the introduction of a second alkyl substituent in the exocyclic amino group (see Fig. 2b). Note, in particular, that the introduction of the second alkyl substituent results in pronounced hyperchromic and bathochromic shifts of the long-wavelength maxima of all three forms of the cytosine ring. Another significant difference between the alkylamino cytosines and cytosine is that the short-wavelength maxima of the cationic forms of the former are about 20% lower than that of the latter.

1-Methyl-4-methylaminocytosine (Fig. 3a) is also strikingly similar to 1-methylcytosine [11], the principal difference also being the more pronounced inflexion of the curve for the neutral form of the former at about 235 mμ. This is again com-

[2] Brookes & Lawley [3] obtained $N_{(3)}$-methylcytidine by treatment of the parent substance with dimethyl sulphate. Curiously enough, no methylation of the ribose moiety was observed (cf. methylation of adenosine with dimethyl sulphate [38]).

Table 2

Spectrophotometrically determined apparent pK *values* (± *0.05* pH *units*) *for alkylamino cytosines and their nucleosides at 22°*

Compound	pK_1 [a]	pK_2 [b]
Cytosine	4.60 [c]	12.4 [d]
Monomethylaminocytosine	4.40 [e]	12.7
Dimethylaminocytosine	4.15 [f]	12.8 [f]
1-Methylcytosine	4.55 [g]	—
1-Methylmonomethylaminocytosine	4.40 [h]	—
1-Methyldimethylaminocytosine	4.20 [i]	—
Cytidine	4.10 [g]	—
Monomethylaminocytidine	3.85 [j]	—
Dimethylaminocytidine	3.70 [k]	—

[a] For protonation of the pyrimidine ring 3 nitrogen (see [6, 9, 37]).
[b] For dissociation of the number 2 carbonyl of pyrimidine ring (see [28]).
[c] From [21]; the value of 4.45 previously given in [28] has been shown to be too low [39].
[d] From [28].
[e] Brown [4] gives 4.55.
[f] Wempen *et al.* [39] report values of 4.25 and 12.3, respectively.
[g] From [11].
[h] Kenner *et al.* [20] report a value of 4.47.
[i] Kenner *et al.* [20] report a value of 4.20.
[j] Wempen *et al.* [39[report 3.92.
[k] Wempen *et al.* [39] report 3.62.

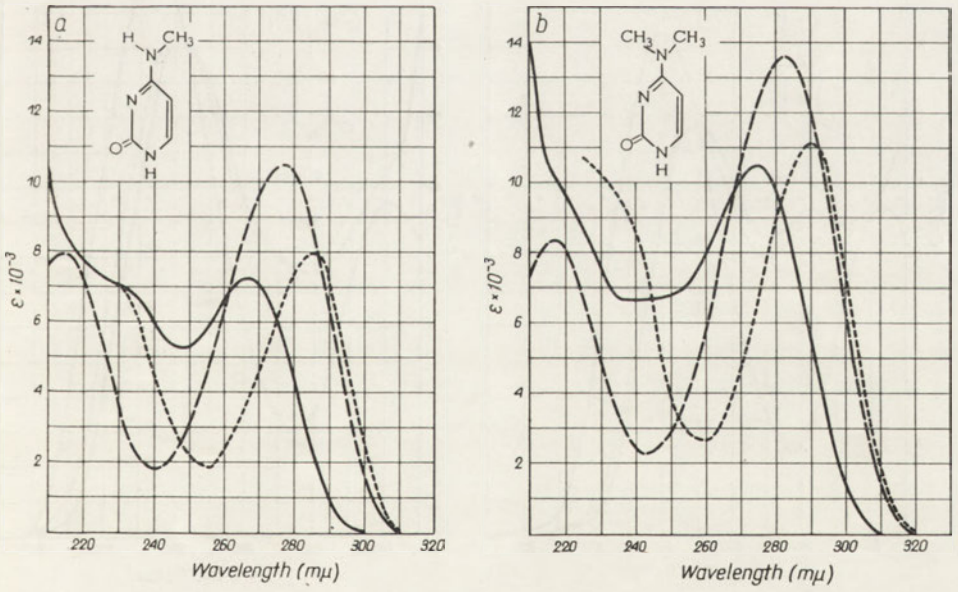


Fig. 2. Absorption spectra of (*a*) monomethylaminocytosine, and (*b*) dimethylaminocytosine: (———), in 0.1 N-HCl, cationic form; (———), in 0.02 M-phosphate pH 7.5, neutral form; (- - -), in 1 N-NaOH, anionic form.

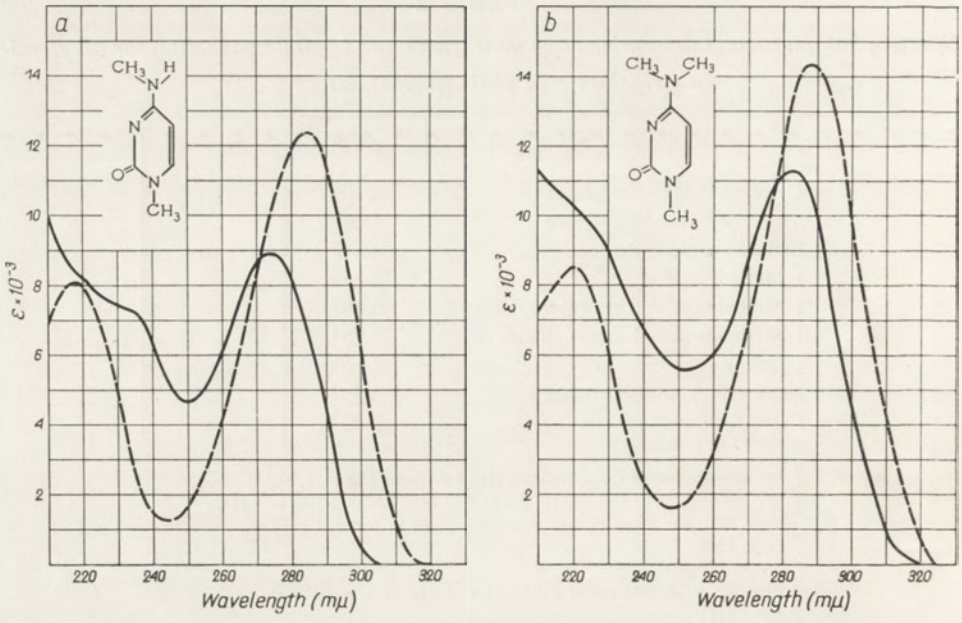


Fig. 3. Absorption spectra of (*a*) 1-methyl-4-monomethylaminocytosine, and (*b*) 1-methyl-4--dimethylaminocytosine: (— — —), in 0.1 N-HCl, cationic form; (———), at pH 7 - 14, neutral form.

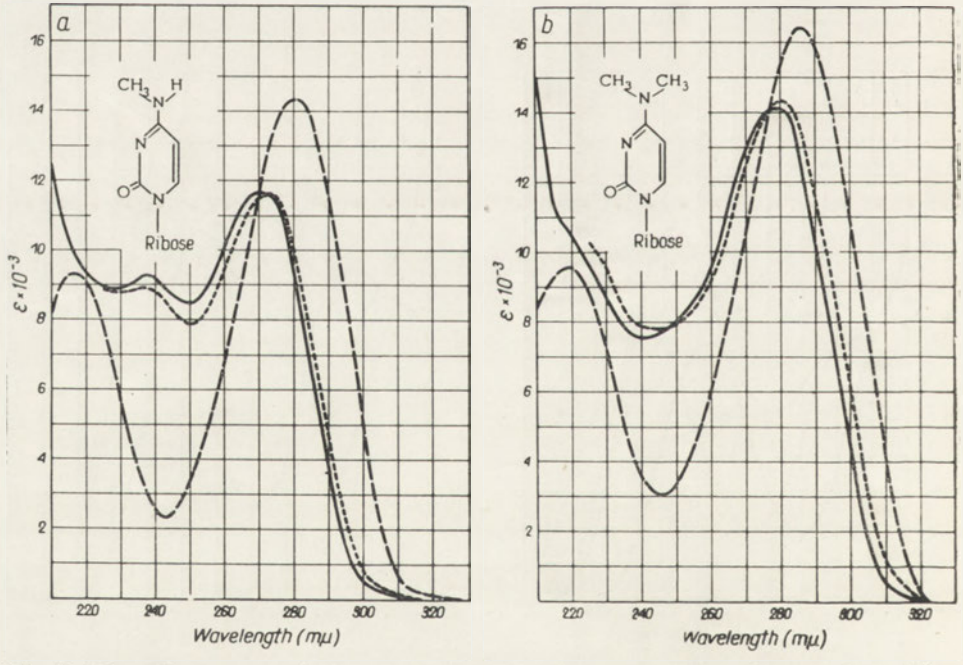


Fig. 4. Absorption spectra of (*a*) monomethylaminocytidine, and (*b*) dimethylaminocytidine: (— — —), in 0.1 N-HCl, cationic form; (———), in 0.02 M-phosphate pH 7.5, neutral form; (- - -), in 1 N-NaOH, neutral form of aglycon, with dissociation of carbohydrate hydroxyls.

pletely eliminated by introduction of a second alkyl substituent in the exocyclic amino group (Fig. 3b). Note also the pronounced increase in extinction of the long-wavelength maxima of both the neutral and cationic forms on introduction of the second alkyl substituent.

Whereas the neutral form of cytidine exhibits a barely perceptible maximum at 230 mμ [11], monomethylaminocytidine possesses a clearly defined second maximum at about 238 mμ (Fig. 4a), which is no longer visible in dimethylaminocytidine (Fig. 4b). This is somewhat analogous to the behaviour of the alkylamino derivatives of 5-fluoro-2′-deoxycytidine derivatives described by Wempen *et al.* [39]. Note again, from Figs. 4a and 4b, the marked bathochromic shifts, and increase in extinction coefficients, of the long-wavelength maxima of both the neutral and cationic forms, due to the second alkyl substituent in the exocyclic amino group. Finally, as in all other nucleosides [11], dissociation of carbohydrate hydroxyls at highly alkaline pH results in slight modifications of the absorption spectra of the neutral forms of the nucleosides (Figs. 4a and 4b).

From Table 2 it will be observed that the successive introduction of one and two alkyl substituents in the exocyclic amino groups of the various cytosine analogues results in small, but definite, decrease in basic strength, as already observed by other observers for similar and analogous derivatives [13, 39].

EXPERIMENTAL

2-Oxy-4-methylaminopyrimidine (*I*) or 4-methylaminocytosine: 250 mg. of 2-oxy-4-ethoxypyrimidine, obtained according to Hilbert & Johnson [15], m.p. 164 - 165°, was dissolved in 7 ml. anhydrous methanol, to which was added 10 ml. of a 30% solution of methylamine in methanol. The reaction mixture was maintained for 18 hr. at 50° in a sealed tube. On cooling, a white precipitate appeared. The tube was opened and the contents evaporated to dryness. The colourless residue, chromatographically homogeneous, was recrystallized from methanol by addition of ethyl acetate, m.p. (decomp.) 272°. Chromatographic and spectral data are given in Table 1 and Fig. 2a, respectively. For $C_5H_7N_3O$ calculated: N, 33.6; obtained: N, 33.8%.

2-Oxy-4-dimethylaminopyrimidine (*II*) or 4-dimethylaminocytosine. This was obtained from 250 mg. 2-oxy-4-ethoxypyrimidine by treatment with a 33% solution of dimethylamine in methanol, as described for I, above. The product was recrystallized from methanol with ethyl acetate, m.p. (decomp.) 251°. Chromatographic and spectral data are given in Table 1 and Fig. 2b, respectively. For $C_6H_9N_3O$ calculated: N, 30.4; obtained: N, 30.1%.

1-Methyl-2-oxy-4-methylaminopyrimidine (*III*) or 1-methyl-4-methylaminocytosine: 250 mg. of 1-methyl-2-oxy-4-ethoxypyrimidine, obtained according to Hilbert & Johnson [16], m.p. 135 - 136°, was dissolved in 5 ml. anhydrous methanol, to which was added 10 ml. of a 30% solution of methylamine in methanol. The reaction mixture was left in a sealed tube for 18 hr. at 50° and then evaporated to dryness to give a colourless residue, chromatographically homogeneous, which was recry-

stallized from methanol and ether, m.p. 180 - 181°. Chromatographic and spectral data are presented in Table 1 and Fig. 3a, respectively. For $C_6H_9N_3O$ calculated: N, 30.4; obtained: N, 29.9%.

1-Methyl-2-oxy-4-dimethylaminopyrimidine (*IV*) or 1-methyl-4-dimethylaminocytosine. This was obtained from 250 mg. 1-methyl-2-oxy-4-ethoxypyrimidine treated with a 33% solution of dimethylamine in anhydrous methanol, as described for III, and recrystallized from methanol and ether, m.p. 178 - 179°. See Table 1 and Fig. 3b for chromatographic and spectral data. For $C_7H_{11}N_3O$ calculated: N, 27.4; obtained: N, 27.2%.

1-(β-D-(2',3',5'-Tribenzoyl)ribofuranose)-4-ethoxypyrimidine (*V*). To 7.55 g. (45 m-moles) of freshly distilled 2,4-diethoxypyrimidine [16] was added a solution of 1-chloro-2,3,5-tribenzoylribose (obtained from 15.3 g., 30 m-moles, of 1-acetylo-2,3,5-tribenzoylribofuranose) in 30 ml. of dry benzene. The benzene was removed under reduced pressure and the reaction flask, fitted with a calcium chloride tube, was placed in a thermostat at 65°. The reaction was allowed to proceed for 48 hr., the temperature being increased at 12-hour intervals until it attained 100°. Light petroleum was added to the resulting dark brown mixture to give a dark viscous precipitate, which was extracted with 5 portions of 300 ml. ethyl ether. The dried residue following extraction, consisting of 520 mg. of dark brown substances not containing the pyrimidine ring, was discarded. The ether extract was concentrated to a light yellow oil, to which was added ethyl acetate and light petroleum (40° - 60°) to turbidity, and then left overnight in the cold room. The oil formed a pseudocrystalline mass over which remained some liquid which was decanted off and which contained unreacted 2,4-diethoxypyrimidine. The residue was dissolved in hot methanol and light petroleum added to turbidity. After 48 hr. at 3°, crystals appeared which, after recrystallization, had a m.p. of 129 - 130°. However, analysis showed the absence of nitrogen and the crystals were probably unreacted 1-acetylo--2,3,5-tribenzoylribose. The crystals were filtered off and the supernatant crystallized, successively from benzene and light petroleum, after prolonged storage in the refrigerator[3] to give 7.1 g. of product (yield 37% with respect to chlororibose, 27% with respect to 2,4-diethoxypyrimidine). Following two recrystallizations from methanol and acetone, the m.p. was 149 - 150°. For $C_{32}H_{29}N_2O_9$ calculated: C, 65.6; H, 4.95; N, 4.8; determined: C, 65.3; H, 4.70; N, 5.1%.

1-(β-D-Ribofuranose)-2-keto-4-methylaminopyrimidine (*VI*) or monomethylaminocytidine: 3 g. of V was dissolved in 20 ml. anhydrous methanol, to which was added 50 ml. of a 30% solution of methylamine in methanol in a sealed tube, which was maintained at 60° for 48 hr. Solvent was removed and the residue dissolved in water and extracted three times with ether. The aqueous layer was evaporated to dryness and the residue crystallized from acetone and methanol. Crystallization was slow; after 5 days at 2°, 480 mg. crystals of VI was filtered off. The filtrate was concentrated to an oil, which was dissolved in 40 ml. water and adsorbed on a

[3] Either crystalline V, or the oil remaining after removal of unreacted 2,4-diethoxypyrimidine, may be used for the methylamination reactions described below.

15×0.8 cm. column of Dowex 50 (H^+). The column was washed with water and eluted with dilute HCl (0.05 M, then 0.075 M). The eluate was concentrated under vacuum to small volume and then distilled with the addition of ethanol and benzene. During distillation the hydrochloride of VI settled out. On recrystallization from 90% ethanol, it melted, with darkening, at 197° and was completely carbonized at 203°. The crude hydrochloride was dissolved in 10 ml. water and passed through a Dowex 2 X8 (OH^-) column. The solution and washings from the column were concentrated to dryness, combined with the 480 mg. precipitate and the whole recrystallized from 80% methanol, to give 1.1 g. (86% theor.), m.p. 237° (darkening, decomp.), carbonized at 243°. For $C_{10}H_{15}N_3O_5$ calculated: N, 16.2; determined: N, 15.9%.

1-(β-D-Ribofuranose)-2-keto-4-dimethylaminopyrimidine (*VII*) or dimethylaminocytidine. This was obtained as for VI, by treatment of V with a 33% solution of dimethylamine in methanol, in essentially quantitative yield as an oil which did not crystallize. It was purified by adsorption on a Dowex 50 (H^+) column and neutralization of the concentrated eluate with some Dowex 2 X8 (OH^-). About 7% of the product was lost through these manipulations. The final product crystallized difficultly from methanol and acetone, m.p. 226° (darkening, decomposition). Part of the product was freeze-dried after purification with activated carbon. For $C_{11}H_{17}N_3O_5$ calculated: N, 15.5; obtained: N, 15.1%.

1-(β-D-(2′:3′-O-Isopropylidene)ribofuranose)-2-keto-4-methylaminopyrimidine (*VIII*). To 515 mg. (2 m-moles) of VI in 74 ml. dry acetone was added 4 g. *p*-toluenosulphonic acid and the reaction mixture kept, with shaking, for 20 hr. at 37°. Solvent was removed and the residue dissolved in 35 ml. water at 0° with addition of about 10 ml. Dowex 2 X8 (HCO_3^-). Following shaking for 30 min., the ion exchanger was filtered off and washed. A chromatographic control at this point showed that the combined filtrate and washings contained about 32% unreacted nucleoside, which was removed with the aid of a column of Dowex 2 X8 in the borate form [7]. The resulting eluate was concentrated and the product crystallized from water to give 380 mg. (65% theor.) which was recrystallized from water and acetone (1 : 1) as white needles, m.p. (decomp.) 281°. For $C_{13}H_{15}N_3O_5$ calculated: N, 14.1; obtained: N, 14.5%.

1-(β-D-(2′:3′-O-Isopropylidene)ribofuranose)-2-keto-4-dimethylaminopyrimidine (*IX*). This was obtained as described above for VIII, in 56% yield, m.p. (decomp.) 267°. For $C_{14}H_{17}N_3O_5$ calculated: N, 13.4; obtained: N, 13.65%.

2′,3′-O-Benzylidenemethylaminocytidine (*X*). To 350 mg. of carefully dried VI suspended in 8 ml. of freshly distilled benzaldehyde was added 0.7 g. of anhydrous $ZnCl_2$ [14]. The mixture was shaken at room temperature for 48 hr., 250 ml. ether added, and the whole left overnight in the cold room. The resulting precipitate was filtered off, washed with ether, and dissolved in 15 ml. methylcellosolve. To this was added 2 ml. 5 N-NaOH, following which gaseous CO_2 was bubbled through the solution until it was neutral. The resulting precipitate was centrifuged off and washed with hot methylcellosolve. The filtrate was brought to dryness, the residue taken up in a minimal volume of ethanol, and water added to give a gelatinous

precipitate. The latter was collected by centrifugation and attempts made to crystallize it from a variety of solvents including ethanol, methanol, acetone, but without success. An aqueous methanolic solution of product was therefore treated with activated carbon, filtered, evaporated under reduced pressure to small volume, and freeze-dried. The product was chromatographically homogeneous in solvent systems *A* and *E*. It was not adsorbed on Dowex 1 X8 (borate form) and was inert to periodate. The overall yield was essentially quantitative, and the product was used as such for phosphorylation (see below).

2',3'-O-*Benzylidenedimethylaminocytidine* (*XI*). This was prepared from 330 mg. VII as described in the preceding section, to give a product in essentially quantitative yield which, however, likewise resisted crystallization. It was chromatographically homogeneous in solvents *A* and *E*, was not adsorbed on Dowex 1 X 8 (borate form), and was resistant to periodate.

Methylaminocytidine-5'-phosphate (*XII*): (a) 50 mg. of 2',3'-*O*-isopropylidenemethylaminocytidine (VIII) was phosphorylated by the "polyphosphate" method [22] and isolated as the barium salt in 39% yield. The product was chromatographically homogeneous in solvents *C* and *D*, and gave a positive periodate reaction. On treatment with prostate phosphomonoesterase it was transformed quantitatively to methylaminocytidine (VI).

(b) 215 mg. (0.6 m-mole) of VIII was phosphorylated by means of β-cyanoethylphosphate according to Tener [36], using a sixfold excess of the phosphorylating reagent. The reaction time was prolonged to 48 hr. with occasional shaking of the reaction vessel. Following alkaline and acid hydrolysis to remove the cyanoethyl and protecting benzylidene groups, paper chromatography demonstrated a 71% yield. The phosphorylated product was isolated by adsorption on Dowex 50 (H^+) and elution with dilute ammonia, and further purified by adsorption on a Dowex 1 X8 (Cl^-) column followed by elution with dilute HCl. The effluent was evaporated to dryness three times, the residue taken up in a minimal quantity of water and precipitated with an excess of ethanol. The resulting product was identical with that described in (a) above.

Methylaminocytidine-5'-pyrophosphate (*XIII*) was prepared from 90 mg. (0.25 m-mole) methylaminocytidine-5'-phosphate as described for cytidine-5'-pyrophosphate *via* the morpholidate derivative [25], in 76% yield. The product was homogeneous in solvent *D* and in the solvent system propan-2-ol - 1% $(NH_4)_2SO_4$ (6 : 4, v/v); it was isolated as the lithium salt and proved active in the polynucleotide phosphorylase system.

Dimethylaminocytidine-5'-phosphate (*XIV*). This was prepared as described above in method (b) for methylaminocytidine-5'-phosphate. The *5'-pyrophosphate* (*XV*) was then prepared in the same way as the pyrophosphate of methylaminocytidine (see preceding paragraph).

2',3'-O-*Benzylidene-5-methylcytidine* (*XVI*). This was obtained from 1 g. of 5-methylcytidine as previously described in 100% yield. The product was crystallized from aqueous ethanol. After several recrystallizations m.p. 184 - 186°. Chromato-

graphically homogeneous with a negative periodate test. For $C_{17}H_{19}O_5N_3$ calculated: N, 12.2; obtained: N, 12.5%.

5-Methylcytidine-5'-phosphate (*XVII*). From 350 mg. (1 m-mole) of XII. Phosphorylation with β-cyanoethylphosphate, and isolation of the product was carried out according to the procedure described by Tener [36]. Yield 230 mg. (free acid) 55%. For $C_{10}H_{16}O_8N_3P$ calculated: N, 12.4; P, 9.2; obtained: N, 12.7; P, 8.6%.

5-Methylcytidine-5'-diphosphate (*XVIII*). From 115 mg. (0.33 m-mole) of XIII. This was obtained and isolated in essentially quantitative yield as described by Moffat & Khorana [25] for cytidine-5'-diphosphate *via* the intermediate morpholidate derivative, to give 125 mg. of $C_{10}H_{14}O_{11}N_3P_2 \cdot Li_3 \cdot 3H_2O$. Calculated: N, 8.6; P, 12.7; obtained: N, 8.8; P, 12.1%.

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REFERENCES

[1] Basilio C. & Ochoa S., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds. Vol. 6, p. 713, Academic Press, New York 1963.
[2] Bredereck H., Haas H. & Martini A. - *Chem. Ber.* **81**, 307, 1948.
[3] Brookes P. & Lawley P. D. - *J. Chem. Soc.*, 1348, 1962.
[4] Brown D. J. - *J. Appl. Chem.* **9**, 203, 1959.
[5] Brown D. J. - Nature **189**, 828, 1961.
[6] Brown D. J. & Lyall J. M. - *Austral. J. Chem.* **15**, 851, 1962.
[7] Chambers R. W., Shapiro P. & Kurkov V. - *J. Amer. Chem. Soc.* **82**, 970, 1960.
[8] Farkaš J., Kaplan L. & Fox J. J. - *J. Org. Chem.* **29**, 1469, 1964.
[9] Fikus M., Wierzchowski K. L. & Shugar D. - *Photochem. Photobiol.* **1**, 325, 1962.
[10] Fox J. J., Yung N., Davoll J. & Brown G. B. - *J. Amer. Chem. Soc.* **78**, 2117, 1956.
[11] Fox J. J. & Shugar D. - *Biochim. Biophys. Acta* **9**, 369, 1952.
[12] Fox J. J., Van Praag D., Wempen I., Doerr I. L., Cheong L., Knoll J. E., Eidinoff M. L., Bendich A. & Brown G. B. - *J. Amer. Chem. Soc.* **81**, 178, 1959.
[13] Fox J. J. & Wempen I. - *Advanc. Carbohyd. Chem.* **14**, 283, 1959.
[14] Gulland J. M. & Smith J. - *J. Chem. Soc.* 1527, 1948.
[15] Hilbert G. E. & Jansen E. F. - *J. Amer. Chem. Soc.* **57**, 552, 1935.
[16] Hilbert G. E. & Johnson T. B. - *J. Amer. Chem. Soc.* **52**, 2001, 1930.
[17] Hilbert G. E. & Johnson T. B. - *J. Amer. Chem. Soc.* **52**, 4489, 1930.
[18] Janion C. & Shugar D. - *Biochim. Biophys. Res. Commun.* **18**, 617, 1965.
[19] Janion C. & Shugar D. - *Acta Biochim. Polon.* **12**, 337, 1965.
[20] Kenner C. W., Reese C. B. & Todd A. R. - *J. Chem. Soc.* 855, 1955.
[21] Levene P. A. - *J. Biol. Chem.* **10**, 229, 1926.
[22] Michelson A. M. - *J. Chem. Soc.* 1957, 1958.
[23] Michelson A. M., *The Chemistry of Nucleosides*. Academic Press, New York - London, 55, 1963.
[24] Miles H. T. - *J. Amer. Chem. Soc.* **79**, 2565, 1957.
[25] Moffat J. G. & Khorana H. G. - *J. Amer. Chem. Soc.* **83**, 649, 1961.
[26] Recondo E. F. & Reyderknecht H. - *Helv. Chim. Acta* **42**, 1171, 1959.

[27] Scannell J. P. & Allen F. W. - *J. Org. Chem.* **25**, 2143, 1960.
[28] Shugar D. & Fox J. J. - *Biochim. Biophys. Acta* **9**, 199, 1952.
[29] Shugar D. & Szer W. - *J. Mol. Biol.* **5**, 580, 1962.
[30] Szer W. - *Biochim. Biophys. Res. Commun.* **20**, 182, 1965.
[31] Szer W. & Ochoa S. - *J. Mol. Biol.* **8**, 823, 1964.
[32] Szer W. & Shugar D. - *Acta Biochim Polon.* **7**, 491, 1960.
[33] Szer W. & Shugar D. - *Acta Biochim. Polon.* **8**, 235, 1961.
[34] Szer W. & Shugar D. - *Biokhimija* **26**, 840, 1961.
[35] Szer W., Świerkowski M. & Shugar D. - *Acta Biochim. Polon.* **10**, 87, 1963.
[36] Tener G. M. - *J. Amer. Chem. Soc.* **83**, 159, 1961.
[37] Ueda T. & Fox J. J. - *J. Amer. Chem. Soc.* **85**, 4024, 1963.
[38] Wacker A. & Ebert M. - *Z. Naturforsch.* **14b**, 709, 1959.
[39] Wempen I., Duschinsky R., Kaplan L. & Fox J. J. - *J. Amer. Chem. Soc.* **83**, 4775, 1961.

SYNTEZA I WŁASNOŚCI NIEKTÓRYCH METYLOWANYCH POCHODNYCH CYTYDYNY, ZWIĄZKÓW POŚREDNICH ORAZ KWASU POLI-5-METYLOCYTYDYLOWEGO

Streszczenie

1. Przeprowadzono syntezy pochodnych metyloaminowych i dwumetyloaminowych: cytozyny, 1-metylocytozyny, cytydyny, 5′-fosforanu cytydyny i 5′-pirofosforanu cytydyny. Wykazano, że dwa ostatnie metylowane analogi cytydyny są substratami dla fosforylazy polinukleotydowej.

2. Badano mechanizm powstawania produktów pośrednich w syntezie nukleozydów.

3. Podano i omówiono widma w ultrafiolecie, wartości p*K* i niektóre własności otrzymanych związków.

4. Przeprowadzono syntezę następujących pochodnych 5-metylocytydyny: 2′,3′-*O*-benzylidenocytydyny, 5′-fosforanu i 5′-pirofosforanu. Ten ostatni okazał się substratem fosforylazy polinukleotydowej.

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Note added in proof: Since submission of the above manuscript, we have been informed by Dr. B. G. Lane of the isolation from ribosomal RNA in low yield of a new nucleoside analogue which exhibited spectral properties corresponding to those in Fig. 4a, above; and, partially on this basis, identified as 2′-*O*-methyl-4-monomethylaminocytidine.

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RECENZJE KSIĄŻEK

Neustadt D. H.: CHEMISTRY AND THERAPY OF COLLAGEN DISEASES (with contributions of J. Rotstein). Charles C. Thomas, Publ., Springfield (Ill.) 1963; str. XI+161, cena 7.50$.

Pojęcie „choroby kolagenowe" czy „kolagenozy" wprowadzone zostało przez Klemperera w 1942 r. Historia tych chorób ma swój początek w obserwacjach Hallera z XVII w. Później badali je But i Schultze w XIX w. i wreszcie Schade w latach dwudziestych i Klinge w latach trzydziestych naszego wieku.

Omawiana książka jest 520-tą pozycją z „American Lectures Series" i przeznaczona jest dla lekarzy praktyków, którzy w codziennej praktyce spotykają się z takimi schorzeniami kolagenowymi jak reumatyzm i inne.

Pierwszy rozdział książki poświęcony został omówieniu statyki biochemicznej tkanki łącznej. Metabolizm tej tkanki jest potraktowany marginesowo. Następne rozdziały omawiają poszczególne kolagenozy. Rozdział drugi poświęcony jest reumatycznemu zapaleniu stawów. Po omówieniu ogólnych założeń biochemicznych, takich jak chemia krwi, fibrynoidu, kwasu hyaluronowego, reakcji immunologicznych i zjawisk alergicznych, Autor dokonuje szerokiego, krytycznego przeglądu metod terapeutycznych. Rozdział drugi jest najbardziej wyczerpująco opracowanym rozdziałem. Następne rozdziały poświęcone są: trzeci — *lupus erythematosus disseminatus*, czwarty — *periarteritis nodosa*, piąty — *polymyositis* i *scleroderma diffusa*, oraz szósty, ostatni — schorzeniom tkanki łącznej z a-γ-globulinemią.

Autor książki kładzie główny nacisk we wszystkich rozdziałach, poza pierwszym, na zagadnienia terapii tych schorzeń, przez co książka staje się cennym vademecum dla specjalisty reumatologa i lekarza zajmującego się schorzeniami tkanki łącznej. Książka zawiera dość obszerny wykaz piśmiennictwa (650 pozycji) doprowadzony do 1961 r. włącznie.

Piękna szata zewnętrzna, zwięzły styl, bardzo przejrzyste ilustracje czynią z tej książki nienużącą lekturę fachową. Jest to niewątpliwie cenna pozycja dla lekarzy praktyków. Biochemika mogą razić pewne naiwności, których przykładem może być choćby „słowniczek" na końcu książki. W tym „słowniczku" Autor w wielu terminach chemicznych upraszcza definicje na swój, nie zawsze zgodny z ogólnie przyjętymi konwencjami, sposób.

Juliusz Popowicz

Kaplan S. A.: GROWTH DISORDERS IN CHILDREN AND ADOLESCENTS. Charles C. Thomas, Publ., Springfield (Ill). 1964; str. 202, cena 8.50 $.

Książka prof. S. A. Kaplana ukazała się w serii „American Lectures Series", której celem jest zapoznawanie szerokich rzesz lekarzy, studentów, biologów, psychologów i wychowawców z postępami biochemii. Omawiana książka jest próbą syntezy współczesnych poglądów na rolę czynników sterujących procesem wzrostu oraz dojrzewania człowieka i przemiany materii — z obserwacjami klinicznymi.

Na podstawie własnego wieloletniego doświadczenia i danych z piśmiennictwa Autor omawia przebieg normalnego wzrostu i dojrzewania dzieci oraz jego warianty fizjologiczne. Przy pomocy wielu rycin i tabeli obrazuje poszczególne etapy rozwoju i przedstawia wskaźniki pozwalające na ich ocenę. S. Kaplan poświęca dużo uwagi współczesnym metodom wyznaczania zakresu normy dla parametrów poszczególnych wartości. Szczególnie podkreśla przydatność określania stopnia dojrzałości układu kostnego dla oceny dynamiki rozwoju danego osobnika i przewidywania, jaki

wzrost osiągnie on ostatecznie. Przy omawianiu czynników mających duży wpływ na procesy wzrostu dużo miejsca poświęcono roli hormonów: hormonu wzrostu, hormonów tarczycy i gruczołów płciowych, z uwzględnieniem współczesnych wyników badań nad hormonem wzrostu i zaburzeniami rozwoju cielesno-płciowego uwarunkowanymi genetycznie (np. w zespole Turnera).

Reasumując należy podkreślić, że książka zaznajamia ogólnie lekarzy i pokrewnych specjalistów z kierunkami współczesnych badań biochemicznych i genetycznych wiążących się z procesem wzrostu, natomiast biochemikowi ukazuje kliniczne aspekty tego procesu.

Alicja Blaim

HUMAN BODY COMPOSITION. (J. Brožek, ed.) Pergamon Press, London, 1965; str. 301+X; cena 80 s.

Omawiana książka przynosi materiały konferencji „Symposia of the Society for the study of human biology", która odbyła się w Londynie w 1963 r., wraz z kilkoma uzupełniającymi rozdziałami pióra wydawcy. Wspomniane Towarzystwo, powstałe w 1958 r. w Londynie, ma na celu studia nad człowiekiem jako gatunkiem, a przede wszystkim nad zmiennością, ewolucją, genetyką, zdolnością adaptacji i ekologią człowieka. Towarzystwo organizuje w ciągu roku dwa sympozja, których wyniki publikuje w wydaniu książkowym. Omawiany tom jest VII tomem tej serii, a konferencja londyńska była trzecią, która za temat miała skład ciała ludzkiego.

Pierwszy referat J. Brožka omawia krytycznie metody jak: antropometryczne pomiary na rentgenogramach, denzytometrię, hydrometrię i ilościowe oznaczanie elektrolitów metodą izotopową.

W następnym referacie E. M. Widdowson z Cambridge przedstawiła wyniki nielicznych dotąd całkowitych analiz zwłok ludzkich. W XIX wieku znane były wyniki trzech niezupełnie kompletnych analiz. Między rokiem 1945 a 1962 przeprowadzono 7 dalszych analiz, z tego 3 są dziełem referentki. Analizy te wykazały, że największe wahania dotyczą zawartości tłuszczu i wody, które stoją do siebie w zależności odwrotnej. J. W. Beeston przedstawił aparat do oznaczania gęstości ciała owcy, oparty na pomiarze przyrostu ciśnienia w zamkniętej przestrzeni po wprowadzeniu odmierzonej ilości wody. Oblicza się stąd objętość, przez którą dzieli się ciężar otrzymując gęstość ciała. A. Pfau z N.R.F. mówił o interpretacji zawartości w mięśniach potasu oznaczonego metodą izotopową. R. W. Parnell z Oxfordu przedstawił korelację między wzrostem, kształtem i składem ciała, podkreślając znaczenie tej korelacji dla statystycznej oceny wyników. Wreszcie J. Durnin z Glasgow dyskutował zagadnienie „jednostki odniesienia" w badaniach nad metabolizmem. Omówione referaty stanowią pierwszą część książki pt. „Approaches".

W części drugiej, mówiącej o zastosowaniach tych badań u zdrowego człowieka, znajdujemy na wstępie dłuższy przegląd historyczny J. Brožka, stanowiący przedruk z Science (tom 134, str. 929, 1961 r.). Następnie R. Passmore z Edynburga dyskutuje rezerwy składników ciała ludzkiego oznaczając dopuszczalną dzienną stratę i czas, na jaki by wystarczyły przy zupełnej karencji danego składnika; wyniki zestawia on w interesującej tabeli. H. Ljunggren ze Szwecji dyskutuje różnice wynikające z płci, wykazując *caeteris paribus* wyższy procent tłuszczu u kobiet. H. B. Young z Bostonu przedstawia porównanie składu ciała dwu grup dzieci włoskich w ojczyźnie oraz dzieci emigrantów w Bostonie, wskazując na różnice wynikające z różnic w stopie życiowej i w sposobie odżywiania. J. Parizkova referuje wpływ zaprawy fizycznej na skład ciała. K. Olesen z Kopenhagi przedstawia system analizy zawartości wody w poszczególnych przestrzeniach (compartments) za pomocą metody rozcieńczeń odpowiednio dobranych izotopów i obliczania na tej podstawie składu ciała. B. Friis-Hansen z Kopenhagi omawia zmiany w zawartości i rozkładzie wody, związane z wzrostem, dojrzewaniem i starzeniem się. Zamyka tę część referat J. M. Tanner z Londynu, omawiający porównawczo skład ciała u dzieci i dorosłych na podstawie pomiarów na radiogramach.

Część III przynosi zastosowania kliniczne. Otwiera ją wstępny artykuł J. Brožka. Następnie J. F. Brock i Nanson, obaj z Południowej Afryki, mówią o znaczeniu odżywiania, a P. Bjurulf z Lund omawia sprawy degeneracji tłuszczowej. Podkreśla on konieczność rozróżnienia między

odkładaniem się tłuszczu w powiększających się komórkach a stanem, w którym gwałtownie rośnie ilość komórek tłuszczowych bez powiększenia rozmiarów poszczególnych komórek. Zamykają książkę dwa „Appendices" pióra wydawcy.

Poświęciliśmy stosunkowo dosyć dużo miejsca omówieniu tej książki, by zwrócić uwagę na tę kształtującą się w naszych oczach nową dziedzinę wiedzy, wypełniającą granice między antropologią, biochemią i naukami medycznymi. Występujący w niej autorzy używają często w jej określeniu nazwy „antropologia fizyczna". Sądzę, że omawiana książka powinna zainteresować wielu biochemików i fizjologów oraz lekarzy.

Józef Heller

Evans R. M.: THE CHEMISTRY OF THE ANTIBIOTICS USED IN MEDICINE. Pergamon Press, London 1965; str. 226, cena 25 s.

Omawiana książka składa się z dziesięciu rozdziałów oraz dwu appendixów. W rozdziale I podano chronologicznie daty odkrycia poszczególnych antybiotyków, metody poszukiwania nowych antybiotyków oraz zestawienie szczepów wytwarzających niektóre z nich. Autor wypowiada przy tym interesującą opinię na temat wielkiej stosunkowo ilości antybiotyków wprowadzonych do lecznictwa w poprzednich latach w porównaniu ze stosunkowo małą ilością w ostatnich latach; twierdzi on, iż nie wynika to z mniejszego zainteresowania antybiotykami lecz dowodzi wzrostu wymagań ze strony klinik i większej specyfiki stosowania antybiotyków w lecznictwie.

Dalsze rozdziały książki omawiają antybiotyki sklasyfikowane na podstawie podobieństw struktury: rozdziały II, III i IV obejmują antybiotyki o strukturze pochodnych aminokwasów, rozdział V — antybiotyki pochodne cukrów, rozdziały VI, VII i VIII — antybiotyki, których struktury dają się wyprowadzić z jednostek octanowych lub propionianowych; rozdział IX obejmuje antybiotyki różne. W rozdziale X podany jest mechanizm działania antybiotyków, w appendix I — dane fizyko-chemiczne antybiotyków omówionych w poprzednich rozdziałach książki. Appendix II poświęcony jest antybiotykom o działaniu przeciwnowotworowym. Na końcu książki umieszczony jest indeks rzeczowy antybiotyków i szczepów; książka nie zawiera indeksu nazwisk.

R. M. Evans przyjmuje klasyfikację antybiotyków podaną przez Abrahama i Newtona w 1960 r. (*Brit. Med. Bull.*, 1960, 16, 3). W opisie każdego antybiotyku podaje rodzaj szczepów wytwarzających dany antybiotyk, jego aktywność *in vitro*, metodę izolowania, strukturę chemiczną i charakterystyczne dla niej reakcje, syntezę, oraz zastosowanie kliniczne tego antybiotyku. W przypadku większości antybiotyków omawiany jest również wpływ zmian w strukturze na zakres i mechanizm działania.

Rozdział II omawia D-cykloserynę i chloramfenikol, a więc antybiotyki, w cząsteczce których występuje tylko jeden aminokwas; rozdział III poświęcony jest penicylinom i cefalosporynom, antybiotykom, w cząsteczce których występują po dwa aminokwasy. W zakończeniu rozdziału III, obejmującego penicyliny i cefalosporyny, Autor uzasadnia podobieństwo struktur tych dwu grup antybiotyków, podając opis i schemat transformacji pochodnej penicyliny α-fenoksymetylowej w analog cefalosporyny C.

We wstępie rozdziału IV omówione są różnice między białkami i hormonami o strukturze polipeptydów a antybiotykami polipeptydowymi, mianowicie występowanie w strukturze antybiotyków „niezwykłych" aminokwasów, jak kwasu α,γ-dwuaminomasłowego i α,β-dwuaminopropionowego, nasyconych kwasów alifatycznych (np. kwasu metylooktanowego) czy też „nienaturalnych" D-aminokwasów. Omawiając zależności strukturowe między poszczególnymi antybiotykami polipeptydowymi Autor zwraca słusznie uwagę, iż takie antybiotyki jak polimiksyny i cyrkuliny, które zawierają w cząsteczce po pięć grup zasadowych, działają na drobnoustroje Gram-ujemne, te zaś, które posiadają mniejszą ilość grup zasadowych w cząsteczce, jak bacytracyna (trzy grupy zasadowe), gramicydyna S (dwie grupy zasadowe), tyrocydyna A (jedna grupa zasadowa) działają na drobnoustroje Gram-dodatnie. Nieaktualną sprawą jest traktowanie gramicydyn A, B i C jako związków o budowie cyklicznej, gdyż stwierdzono ostatnio, że w cząsteczkach tych związków nie występują wolne grupy aminowe nie dlatego, że budowa ich jest cykliczna,

lecz dlatego, że grupy te zablokowane są grupą formylową. Podobnie przedstawia się sprawa wiomycyny, dla której podano ostatnio dwie propozycje struktury (Bowie i współprac., 1964, Dyer i współprac., 1965).

Rozdział V omawia antybiotyki pochodne cukrów, a więc streptomycyny, neomycyny i paromomycyny oraz kanamycyny. Na wstępie Autor podkreśla wspólne cechy tych antybiotyków, następnie wiele uwagi poświęca chemii tych związków oraz stereochemii ich produktów degradacji. Bardzo przejrzyście podane są podobieństwa i różnice między neomycyną B i paromomycyną I oraz neomycyną C i paromomycyną II.

Rozdziały VI, VII i VIII omawiają antybiotyki, których jednostkami strukturowymi są octany lub propioniany, przy czym rozdział VI obejmuje antybiotyki, w strukturze których występują układy skondensowanych pierścieni (fused-ring systems), a więc tetracykliny, gryzeofulwinę i kwas fusydowy; rozdział VII obejmuje antybiotyki z grupy makrolidów (erytromycynę, karbomycynę, oleandomycynę i spiramycyny) z uwzględnieniem biogenezy tych związków; rozdział VIII obejmuje antybiotyki z grupy polienów (nystatyna, amfoterycyna B i fumagilina). Część rozdziału VI dotycząca tetracyklin omawia chemię tych związków i produkty ich degradacji alkalicznej i kwaśnej oraz katalitycznej redukcji. W oddzielnym podrozdziale podany jest przegląd prób syntezy i przebieg syntezy 6-desmetylo-6-dezoksytetracykliny oraz mieszanej syntezy chemiczno-biologicznej 7-chloro-6-desmetylotetracykliny. Oddzielne miejsce zajmuje biogeneza tetracyklin oraz zagadnienie wpływu struktury chemicznej na aktywność biologiczną zilustrowane tabelarycznie. W dalszej części tego rozdziału omówiona jest gryzeofulwina, z podaniem jej aktualnego wzoru stereochemicznego, syntezy w oparciu o benzofenony i kumarony, biogenezy oraz typowych reakcji chemicznych. Kwas fusydowy omówiony jest głównie w aspekcie prac nad ustaleniem jego struktury stereochemicznej. Autor zwraca dalej uwagę, iż kwas fusydowy wykazuje niską toksyczność przy stosowaniu *per os* i jest dobrze resorbowany z przewodu pokarmowego, a przy tym nie wykazuje oporności krzyżowej z innymi antybiotykami, dzięki czemu jest stosowany głównie w zakażeniach gronkowcowych.

W rozdziale VII, poświęconym makrolidom, Autor przypomina na wstępie, iż są to związki których cząsteczka składa się z makrocyklicznego pierścienia laktonowego oraz z aminocukru związanego z nim glikozydowo i z cukru obojętnego, który związany jest z pierścieniem lub z aminocukrem. Podaje strukturę dezozaminy, występującej w cząsteczce erytromycyny i oleandomycyny, mykozaminy (karbomycyna i spiramycyny) oraz 5-dwumetyloamino-6-metylo-2-hydroksypiranu (spiramycyny), a także obojętnych cukrów: mykarozy (karbomycyna i spiramycyny), kladinozy (erytromycyna) i oleandrozy (oleandomycyna). Szczegółowo omówiona jest struktura erytromycyn, karbomycyny, oleandomycyny i spiramycyn, a także biogeneza tych związków głównie w oparciu o prace Woodwarda i Grisebacha oraz Hofheinza.

Rozdział VIII omawia antybiotyki polienowe: nystatynę, amfoterycynę B i fumagilinę; Autor wyraża opinię, że polieny o działaniu przeciwgrzybowym, które są nienasyconymi makrolidami mają ten sam model strukturalny jak makrolidy omówione w rozdziale VII (nystatyna i amfoterycyna B; fumagilina wykazuje głównie działanie przeciwpełzakowe, ma strukturę polienu lecz nie jest makrolidem). Schemat omawiania tych trzech antybiotyków jest taki sam jak pozostałych. Autor zwraca uwagę, iż nystatyna stosowana jest głównie w przypadkach infekcji grzybiczej, wynikłej wskutek stosowania antybiotyków o szerokim spectrum.

W rozdziale IX omówione są antybiotyki różne, do których R. M. Evans zalicza nowobiocynę, której cząsteczka zawiera fragment o budowie heterocyklicznej, związany z jednej strony z cukrem o budowie L-liksozy , z drugiej z podstawionym kwasem benzoesowym; rystocetyny; wankomycynę i puromycynę, której cząsteczka składa się z 6-dwumetyloaminopuryny, 3-dezoksy-3-amino-D--rybozy i *O*-metylotyrozyny. Omówiona jest biogeneza nowobiocyny i zastosowanie kliniczne.

Ostatni rozdział książki (X) omawia sposób i miejsce działania antybiotyków. Na podstawie tych kryteriów Autor wprowadza podział na trzy grupy: antybiotyki działające na ściankę komórkową (cell wall), działające na błonę komórkową (cytoplasmic membrane) i trzecia grupa, ingerujące w syntezę białek.

Każdy rozdział książki zakończony jest piśmiennictwem. Pozycje piśmiennictwa cytowane są w kolejności podawania ich w tekście i obejmują na ogół informacje do roku 1964 włącznie.

Zuzanna Kowszyk-Gindifer

Ziegler E.: THE REDOX POTENTIAL OF THE BLOOD *IN VIVO* AND *IN VITRO*. C. Thomas Publ., Springfield (Ill.) U.S.A. 1965; stron 196, cena 8.50 $.

Pomiar potencjału oksydoredukcyjnego krwi może być dokonywany w nieuszkodzonej, żywej tkance i dlatego wydaje się, że znaczenie tego badania będzie w przyszłości wzrastać.

Książka E. Zieglera omawia w sposób krytyczny metody oznaczania potencjału oksydoredukcyjnego krwi i wyniki tych pomiarów uzyskiwane przez różnych autorów. Praca składa się z następujących rozdziałów: I. Dotychczasowe pomiary potencjału oksydoredukcyjnego surowicy, osocza i krwi. II. Podstawy fizyko-chemiczne pomiarów potencjału oksydoredukcyjnego: Termodynamiczne i biologiczne znaczenie potencjału oksydoredukcyjnego. III. Oznaczanie potencjału oksydoredukcyjnego krwi *in vivo* i *in vitro*. Nowa metoda pomiaru. IV. Dyskusja i podsumowanie. Pracę kończy rozdział napisany przez prof. J. Rehna: Znaczenie kliniczne potencjału oksydoredukcyjnego krwi. Zebrane przez Autora piśmiennictwo zawiera 93 pozycje.

Praca jest pierwszą wyczerpującą monografią dotyczącą potencjału oksydoredukcyjnego krwi. W książce obok zebrania i krytycznej oceny dotychczasowego piśmiennictwa wiele miejsca poświęcono teoretycznym podstawom pomiarów i dyskusji błędów będących przyczyną dużej rozbieżności dotychczasowych wyników, a następnie wnikliwie i przekonywająco omówiono proponowaną przez Autora metodę pomiaru. Autor stwierdza, że dotychczasowe pomiary były zawsze obarczone dodatnim błędem wskutek adsorpcji tlenu na powierzchni elektrody platynowej wprowadzanej do krwi, w wyniku czego elektroda taka stawała się „elektrodą tlenową". Błędu tego można uniknąć przez polaryzację elektrody platynowej przed przeprowadzeniem pomiaru. Autor wykazuje, że pomiary przy zastosowaniu nowej metody dają wyniki powtarzalne i zgodne z teoretycznymi przewidywaniami. Ciekawe są zwłaszcza wyniki pomiarów dokonywanych *in vivo* na zwierzętach po podaniu dożylnym szeregu środków o znanym potencjale oksydoredukcyjnym. Jeżeli pomiarów takich dokonywać przy użyciu elektrody platynowej niespolaryzowanej, to wyniki różnią się znacznie od przewidywanych teoretycznie. Zastosowanie proponowanej przez Autora elektrody spolaryzowanej powoduje, że wyniki stają się zgodne z przewidywanymi. Z pomiarów dokonywanych przez Autora wynika, że głównym układem oksydoredukcyjnym krwi jest układ kwas askorbinowy/kwas dehydroaskorbinowy.

Praca jest napisana ciekawie i pobudza do myślenia.

Jerzy Umiastowski

SELECTED CONSTANTS OPTICAL ROTATORY POWER. Ia Steroids. (J. Jacques, H. Kogan, G. Ourisson & S. Allard, eds.) Pergamon Press, Oxford 1965; str. 15+1031; cena 370 s.

Książka stanowi czternastą kolejną pozycję z serii Tabel wydawanych od 1947 r. pod patronatem International Union of Pure and Applied Chemistry (IUPAC). Pozycja Nr 6 z tej serii wydana w 1956 r. przedstawia dane skręcalności właściwej 8 tysięcy sterydów poznanych do 1953 r. Obecna praca obejmuje ponad 21 tysięcy związków sterydowych poznanych do roku 1961. Tak wielki rozwój chemii tych związków wielocyklicznych tłumaczy skok ilościowy, jaki obserwujemy między pierwszym a obecnym wydaniem.

We wstępie dwujęzycznym (francuskim i angielskim) podano informacje dotyczące nomenklatury w myśl zaleceń Podkomisji IUPAC do spraw Nomenklatury Sterydów. Stanowi to klucz, którego Autorzy trzymają się konsekwentnie, a który znakomicie ułatwia czytelnikowi poruszanie się wśród tysięcy nazw związków wielocyklicznych.

Książkę podzielono na trzy działy: jeden (778 stron) zestawia w jednolicie opracowanej tabeli najważniejsze dane poszczególnych sterydów, drugi (94 strony) obejmuje bibliografię, a trzeci (144 strony) stanowi indeks omawianych związków. W dziale pierwszym związki uszeregowano

według wzrastającej liczby atomów węgla. Listę otwiera grupa sterydów o 15 atomach węgla, a zamyka ją związek z liczbą atomów węgla równą 97. Każda pozycja tabeli obejmuje kolejno: wzór sumaryczny; nazwę związku; temperaturę topnienia; masę cząsteczkową; rozpuszczalnik, w którym mierzono aktywność optyczną, oraz warunki pomiaru, jak stężenie badanego związku, temperaturę i długość fali; wreszcie skręcalność właściwą i odnośnik do piśmiennictwa. Nazwy związków są nieraz bardzo skomplikowane, co utrudnia czasem odnalezienie pozycji szukanego związku, wymaga jednakże tego złożoność samych sterydów. Dążenia IUPAC zmierzające do ujednolicenia nomenklatury zostały w omawianej książce praktycznie zrealizowane. Usystematyzowanie tej olbrzymiej grupy związków wymagało dużego nakładu pracy i Autorom należą się wyrazy uznania.

Znaczenie książki sięga dalej, aniżeliby to wynikało z jej tytułu. Tabela stałych łącznie z indeksem i bibliografią służy kilku celom, z których może nie najważniejsze są dane skręcalności właściwej oraz temperatury topnienia. Indeks związków, chociaż objętościowo ustępuje tabeli stałych, wydaje się być równie wartościowy dzięki starannie opracowanej systematyce sterydów podającej, obok nazw tradycyjnych, jednolitą nomenklaturę wszystkich sterydów poznanych do 1961 r.

Książka, chociaż stosunkowo droga, winna znaleźć się we wszystkich bibliotekach chemicznych i biochemicznych.

Ryszard Niemiro