

POLSKA AKADEMIA NAUK
KOMITET BIOCHEMICZNY I BIOFIZYCZNY

POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA
BIOCHIMICA POLONICA

QUARTERLY

Vol. XI

No. 2-3

WARSZAWA 1964

PAŃSTWOWE WYDAWNICTWO NAUKOWE

<http://rcin.org.pl>

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Państwowe Wydawnictwo Naukowe — Warszawa, Miodowa 10

Nakł. 1920+150 egz. Ark. wyd. 26,75 ark. druk. 23,25+1,125+0,25.

Papier druk. sat. kl. III, 80 g. 70×100

Oddano do składania 6.IV.1964. Podpisano do druku 23.IX.1964.

Druk ukończono w październiku 1964.

Zam. 186/64

Z-28

Cena zł 50.—

Warszawska Drukarnia Naukowa — Warszawa, Śniadeckich 8



BOLESŁAW SKARŻYŃSKI
1901-1963

A small group of biochemists who survived the war in Poland or returned from abroad, undertook the reconstruction of biochemistry in this country. At that time, their total number was not higher than the number now to be found in any Polish town with an institution of higher education.

The late Professor Dr. Bolesław Skarżyński (who died on March 17, 1963, at the age of 62), was one of the greatest contributors to the work. His uncommon abilities, great industriousness, didactic gifts, and his warm friendliness for young people lent added effect to his work. The influence of his creative, dynamic personality radiated not only on his close collaborators; almost every biochemistry institution in Poland received his help in the training of biochemists. His appeal made at the 1st Congress of Polish Science, in 1950, brought significant results. He sounded the alarm on the work conditions prevailing in Polish biochemistry, and his urging did not go unheeded. Bolesław Skarżyński must be credited with a major part of what has been done in Poland since the war in the organization of science and scientific research. This found acknowledgment by all Polish biochemists in his election as first Chairman of the Polish Biochemical Society.

The Committee of Biochemistry and Biophysics of the Polish Academy of Sciences and the Editors of *Acta Biochimica Polonica* by this collection of works by Professor Skarżyński's friends and closest collaborators, express their profound gratitude for his benevolent spirit which was redolent in his ever-willing help, his creative organization and his original research. With the tribute paid by this publication, we also express our deepest sorrow that he was not permitted for longer to stay among us.

IRENA MOCHNACKA, WŁODZIMIERZ MOZOŁOWSKI, Editors
WŁODZIMIERZ OSTROWSKI, TADEUSZ SZCZEPKOWSKI, MIROSLAWA WEBER, Organizational Committee
JÓZEF HELLER, Chairman, Committee of Biochemistry and Biophysics of the Polish Academy of Sciences

A small group of students who were interested in the subject of the history of the United States, and who were also interested in the history of the world, were organized into a club. The club was organized by the students themselves, and they met regularly to discuss the history of the United States and the world.

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S. LUDEWIG and A. CHANUTIN

EFFECT OF SALTS AND pH ON THE ORGANIC AND INORGANIC PHOSPHATES IN HUMAN RED CELL HAEMOLYSATE**Biochemical Laboratory, University of Virginia Medical School, Charlottesville, Virginia, U.S.A.*

The ability of proteins, particularly plasma albumin, to interact with inorganic and organic compounds has been amply demonstrated [5]. Few data are available concerning such interactions with haemoglobin. Recently, Debro & Lee [3] presented evidence to indicate that adenosine triphosphate reacts with haemoglobin and other proteins to form precipitates when the pH is on the acid side of the isoelectric point. Other triphosphates of purine and pyrimidine ribosides react similarly but diphosphate and monophosphate derivatives are not effective as precipitants. The present investigation deals with conditions which influence the binding between haemoglobin and erythrocyte inorganic phosphate and phosphorylated organic compounds. This study is concerned with the inorganic and organic phosphate concentrations of haemolysates after dialysis against a variety of solutions.

METHODS

Bloods from healthy male medical students were collected in acid-citrate-dextrose and stored immediately at 4° in a cold room where all subsequent steps including washing of erythrocytes, haemolysis, centrifugation and dialysis were done. Each sample of blood (6 ml.) was centrifuged at low speed, the plasma and buffy coat were removed, and the erythrocytes were washed three times with 0.9% NaCl. The washed cells were haemolysed with a small volume of water and the haemolysate was centrifuged at 7700 g for an hour to remove the stroma. The clarified haemolysate was adjusted to 2% haemoglobin by addition of water.

The haemolysates (10 ml.) were bagged in Visking tubing and dialysed against two 4-liter batches of distilled water, NaCl solutions or diethylbarbiturate buffers for 20 hr. When cacodylate buffers were used, the

* Supported by the U.S. Army Medical Research and Development Command, Office of the Surgeon General.

haemolysate was dialysed against two 250-ml. portions for three hour periods and against 1500 ml. for 15 hr. In order to vary the pH, haemolysates were dialysed against two 4-liter portions of dilute, unbuffered HCl or NaOH solutions (pH 4 - 11) during a 20 hr. period. During dialysis the media were stirred continuously by a magnetic stirrer.

Haemoglobin (Hb) concentration was determined by a cyanmethaemoglobin procedure [2] and the phosphates by the Fiske-Subbarow method [4]. The haemolysate was deproteinized with 10% trichloroacetic acid (TCA) and inorganic phosphate (P_i) was determined at once. The total phosphate of the TCA filtrate was determined after digestion by Bartlett's procedure [1]. It is important to maintain the oven at $152 \pm 2^\circ$ particularly if cacodylic acid is present. Filtrates containing diethylbarbiturate buffer require frequent additions of superoxol to decolorize the digest. The organic phosphate (P_{org}) was obtained by subtracting the μ moles P_i /g. Hb from the μ moles total phosphate/g. Hb.

RESULTS

Dialysis of haemolysates of stored blood against water. A single blood was stored in the cold and at weekly intervals samples were removed for the preparation of haemolysates. It is well known that the P_{org} decreases and the P_i increases during storage of blood [7]. In Fig. 1, the

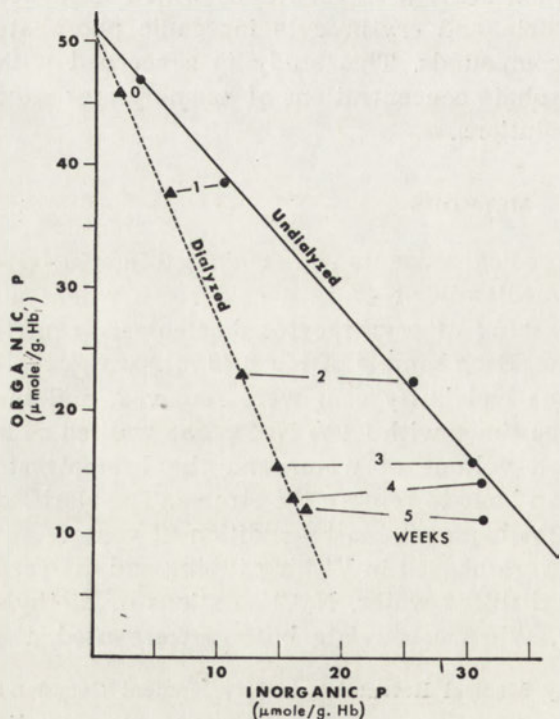


Fig. 1. The relationship between P_i and P_{org} concentrations of red cell haemolysates of stored blood before and after dialysis against water.

plots of the P_{org} against the P_i concentrations yield straight lines for the dialysed and non-dialysed haemolysates with slopes of -1.14 and -2.31, respectively. According to these data, about 50% of the inorganic phosphate is lost during dialysis while the organic phosphate values remain unchanged.

Effect of dialysis against a cacodylic buffer. This buffer was studied because it is used frequently for electrophoretic analysis of erythrocyte haemolysates and of haemoglobin. In this laboratory the cacodylate buffer employed for electrophoresis is composed of sodium cacodylate (0.033 M) and cacodylic acid (0.017 M) at pH 6.5. Electrophoresis of haemolysate in this buffer yields a complex pattern containing at least four components. During the storage of blood, the concentration of one of the components (B) decreases.

A sample of blood was stored in the cold for five weeks and at weekly intervals haemolysates were prepared and dialysed against cacodylate buffer and against water. The pH values for both dialysed haemolysates were 6.5. The data in Fig. 2 show that the P_{org} values decrease during

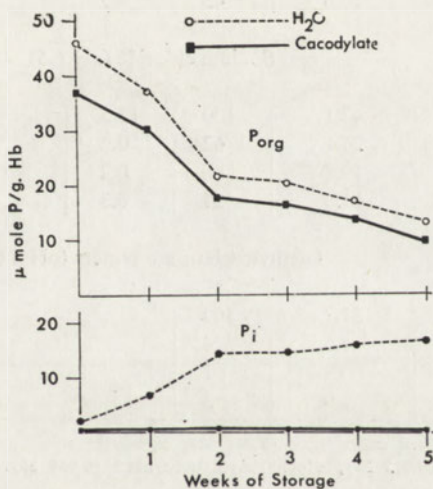


Fig. 2. P_i and P_{org} concentrations of red cell haemolysates of stored blood after dialysis against water and cacodylate buffer.

storage and that the cacodylate-haemolysate values are about 20% lower than those of the water-haemolysates. These changes in concentration during storage approximate those observed for component B. The P_i values of the water-haemolysates increase during storage while only a trace of P_i is present in the cacodylate-haemolysate.

Effect of ionic strength. Results obtained after dialysing haemolysates against cacodylate buffers, NaCl and diethylbarbiturate buffers with ionic strength of 0.033, 0.066 and 0.10 are shown in Table 1.

Table 1

The inorganic and organic phosphate concentrations of haemolysates dialysed against cacodylic buffers, sodium chloride solutions and diethylbarbiturate buffers of different ionic strengths

Molar ionic strength (<i>I</i> /2)	0-day-old blood			28-day-old blood		
	<i>P</i> _i	<i>P</i> _{org}	<i>P</i> _{org} (%)	<i>P</i> _i	<i>P</i> _{org}	<i>P</i> _{org} (%)
	(μmoles/g. Hb)			(μmoles/g. Hb)		
	Cacodylate buffers (pH 6.5)					
H ₂ O,Control	4.0	44.1	100	14.9	14.7	100
0.033	1.4	34.4	78	0.5	11.1	74
0.066	1.1	31.7	72	1.0	9.9	67
0.10	1.4	25.5	58	0.4	9.3	63
H ₂ O,Control	2.8	39.5	100	13.3	19.4	100
0.10	0.4	24.2	61	0.0	13.1	68
0.10 (NaCl)*	0.4	17.6	45	0.7	10.2	53
	Sodium chloride (pH 6.5)					
H ₂ O,Control	2.2	42.1	100	13.5	12.3	100
0.033	0.5	26.1	62	0.9	6.8	55
0.066	0.5	19.8	46	0.2	5.5	45
0.10	0.5	11.5	27	0.3	4.2	34
	Diethylbarbiturate buffers (pH 8.6)					
H ₂ O,Control	3.5	37.7	100			
0.033**	0.1	1.5	4			
0.066	0.5	0.3	1			
0.10	0.1	1.0	3			

* NaCl (0.08 M); sodium cacodylate (0.021 M), cacodylic acid (0.0083 M) [6].

** Diethylbarbiturate (0.033 M), diethylbarbituric acid (0.005 M).

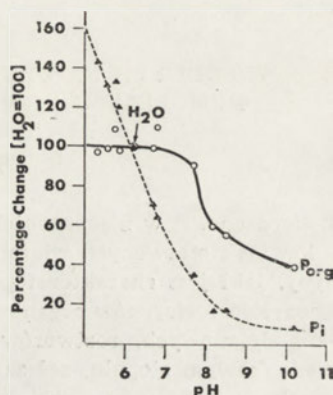
After the dialysis of haemolysates against these solutions, only a trace of P_i is present. The variations in the P_i values have no significance because the analytical procedure is not sufficiently sensitive at low concentrations.

Cacodylate. Two different samples of bloods (0 and 28 days old) were dialysed against cacodylate buffers of different ionic strengths. A progressive loss of P_{org} is noted as the ionic strength increases from 0.033 to 0.1. Dialysis of a haemolysate against a cacodylate buffer containing NaCl [6] with an ionic strength of 0.1 is responsible for an appreciably greater loss of P_{org} than is observed with the unsupplemented 0.1 cacodylate buffer.

Sodium chloride and diethylbarbiturate buffers. Increasing concentrations of NaCl caused progressively greater loss of P_{org} in the haemolysates of both the 0 and 28-day-old bloods. At a relatively low ionic strength of NaCl (0.10) as much as 70% of the P_{org} is lost. Practically all of the P_{org} disappears, after dialysis against diethylbarbiturate buffers (pH 8.6). Experiments with tris buffers at pH 8.6 yield results almost identical with those observed with diethylbarbiturate buffers. This marked decrease cannot be attributed entirely to the pH since dialysis of a haemolysate against dilute sodium hydroxide at pH 8.6 is responsible for a decrease of about 50% of the organic phosphate (Fig. 3).

Effect of pH. The results obtained after dialysis of aliquots of a haemolysate against weak hydrochloric acid and sodium hydroxide solutions are shown in Fig. 3. The values for P_i and P_{org} concentrations are com-

Fig. 3. Percentage changes of P_i and P_{org} concentrations of a red cell haemolysate after dialysis against dilute acid and alkaline solutions. (The pH values of the haemolysates were obtained after dialysis).



pared with those obtained after dialysis against water (pH 6.4). The P_i values are progressively greater as the pH of the dialysed haemolysate decreases to 5.3 and drops steadily as the pH increases to 10.2. At pH 8.0 about 80% of the P_i is lost. The P_{org} concentrations of the dialysed haemolysates remain constant between pH 5.3 and 6.8 but decrease rapidly as the pH increases. At pH 10.2 about 60% of the organic phosphate is lost.

Effect of prolonged dialysis. It was found that the P_i and P_{org} concentrations remain unchanged after dialysing haemolysate against water for three days but there is a gradual decrease in the P_{org} values when dialysed against cacodylate during this period.

SUMMARY

It has been shown that inorganic and organic phosphates of red cell haemolysates are bound to haemoglobin in the absence of salts. No evidence is available to indicate the type of interaction.

The organic phosphate concentration of haemolysates decreases at different rates after dialysis against cacodylate buffers, sodium chloride solutions and diethylbarbiturate buffers. Inorganic phosphate is present in trace amounts after dialysis against these salt solutions.

REFERENCES

- [1] Bartlett G. R. - *J. Biol. Chem.* **234**, 466, 1959.
- [2] Crosby W. H., Munn J. I. & Furth F. W. - *U.S. Armed Forces Med. J.* **5**, 693, 1954.
- [3] Debro J. R. & Lee M. - *Nature*, **190**, 1008, 1961.
- [4] Fiske C. H. & Subbarow Y. - *J. Biol. Chem.* **66**, 375, 1925.
- [5] Goldstein A. - *J. Pharmacol. Exp. Therap.* **95**, 102, 1949.
- [6] Pauling L., Itano H. A., Singer J. & Wells I. C. - *Science* **110**, 543, 1949.
- [7] Rapoport S. - *J. Clin. Invest.* **26**, 591, 1947.

WPLYW SOLI I pH NA ORGANICZNE I NIEORGANICZNE FOSFORANY HEMOLIZATU KRWINEK CZERWONYCH CZŁOWIEKA

Streszczenie

Stwierdzono, iż w nieobecności soli nieorganiczne i organiczne fosforany hemolizatów krwinek czerwonych wiążą się z hemoglobina. Brak jest danych, które wskazywałyby, jaki jest charakter tego wiązania.

Zawartość fosforanów organicznych w hemolizatach dializowanych wobec buforów kakodylanowych, roztworów chlorku sodu i buforów weronałowych spada w niejednakowym stopniu, zaś zawartość fosforanu nieorganicznego spada do ilości śladowych.

Received 27 June 1963.

J. KAWIAK

ARYLSULPHATASE C ACTIVITY IN RAT CARTILAGE*Department of Histology and Embryology, Medical School, Warszawa*

The presence of arylsulphatase has been demonstrated in various organs and tissues, e.g. in the liver, kidney, adrenals and muscles [2, 3]. However, no reports concerning the presence of arylsulphatase activity in cartilage were found in the literature, although the intensive metabolism of sulphates in this tissue would justify such studies. It was the aim of the present work to determine the arylsulphatase activity in epiphyseal cartilages.

MATERIALS AND METHODS

Reagents. 0.05 M-tris (L. Light & Co. Ltd., Colnbrook, Bucks, England) - acetate buffer of appropriate pH was prepared a few days before the experiments. *p*-Nitrophenol (Fabryka Odczynników Chemicznych, Gliwice, Poland, sample no. 973) had absorption maxima at 400 and 230 m μ . Potassium *p*-nitrophenyl sulphate (NPS) was prepared from *p*-nitrophenol and chlorosulphonic acid according to the procedure of Burghardt & Lapworth [1], in the modification of Dodgson & Tudball [8]. In the preparation the nitrogen to sulphur ratio was 2.28 (theoretical value 2.29), the nitrogen being assayed by the Kjeldahl method and sulphur gravimetrically as barium sulphate [12]. Tests for chloride and inorganic sulphate were negative. The purity was assayed spectrophotometrically in a range of 230 - 450 m μ , E_{\max} being at about 285 m μ . The absorption spectra of NPS and *p*-nitrophenol are presented in Fig. 1. Trypan blue and neutral red were R.A.L., Kuhlmann (Paris) products. Lissamine green was obtained through the kindness of Professor Dr. H. Hydén (Göteborg, Sweden).

Cartilage preparation. Male and female albino rats, 14-20 days old, weighing 19-28 g. were used. Following decapitation of animals the femoral bones were quickly isolated and stored in ice-cold distilled water. Distal and proximal cartilage epiphyses were cut off in separate vessels

and left in ice-cold water for 2-4 hr. During this period the water was changed twice. Then the cartilage was weighed and transferred to two changes of cooled to -20° acetone, 10 ml. of acetone per 1 g. of fresh tissue being used. The cartilage was then dried at room temperature (18° , 1 hr.), ground in the mill and stored over CaO at 4° . A part of the material was isolated while the bones were immersed in ice-cold isotonic (0.25 M) saccharose solution or in water, and was used for experiments without further treatment.

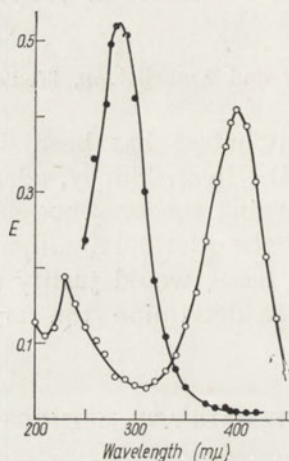


Fig. 1. Absorption spectrum of (O) *p*-nitrophenol and (●) *p*-nitrophenyl sulphate dissolved in 66.6% ethanol with 0.166 N-NaOH (read against the same mixture).

For comparative experiments liver acetone powder was used. The livers from 14 day-old rats were stored for 2-4 hr. in ice-cold water, then homogenized and dried with cold acetone.

Assay procedure. For glass washing, mixtures containing sulphuric acid were never used. For the enzymic assays the following procedure was adopted: 15 mg. portions of acetone-dried cartilage preparation were weighed in centrifuge tubes and suspended in 0.5 ml. of 0.05 M-tris-acetate buffer, pH 7.5. After a preincubation period of 4 min. at 37° , 2.5 μ mole NPS in 0.5 ml. of the same buffer solution was added and incubation was continued for 1 hr. at 37° . The reaction was stopped by immersion of the tubes in ice water and by addition of 4 ml. 95% ethanol. The precipitated proteins and carbohydrates as well as the suspended cartilage preparation were removed by centrifugation. To 4 ml. of the supernatant 0.8 ml. of 1 N-NaOH was added and extinction was read at 400 mμ in 1 cm. cuvettes (Hilger spectrophotometer Uvispek) against 66.6% ethanol (v/v) containing 0.166 N-NaOH. The enzyme acti-

vity was expressed as μ moles of liberated *p*-nitrophenol. All assays were made in duplicate and experiments were repeated 2 - 3 times. As control served samples to which cartilage was added after incubation. Similar procedure was applied in experiments with fresh cartilage isolated in isotonic saccharose solution or in water, except that 50 mg. portions of the cartilage were taken for every sample and cartilage isolated in 0.25 M-saccharose solution was incubated in a medium to which saccharose was added to the same concentration (isotonic medium).

The recovery of *p*-nitrophenol was tested by incubating the cartilage in the medium containing 0.18 μ mole *p*-nitrophenol instead of NPS. The recovery of *p*-nitrophenol from samples incubated with acetone-dried cartilage and with fresh cartilage in 0.25 M-saccharose was practically complete (99%); with fresh cartilage isolated and incubated in hypotonic medium it was less satisfactory (97%).

Supravital staining. Survival of the cartilage incubated in the isotonic solution was controlled by staining with 0.005% neutral red solution, for 10 min. at 37°. Moreover, tests with 0.05% trypan blue and 0.1% lissamine green solutions (2 to 3 min. at room temperature) were applied. All dyes were dissolved in buffered Ringer-Krebs solution, pH 7.4.

RESULTS

Incubation of NPS with the suspension of acetone-dried cartilage lead to the release of *p*-nitrophenol (Fig. 2). The solubility of the enzyme appeared to be very low. The suspension of acetone-dried cartilage in 0.05M-tris - acetate buffer, pH 7.5, was left for 1 hr. at 4°, then centrifuged and in the supernatant only about 3% of enzymic activity was found. These results suggest the presence of the insoluble arylsulphatase C [4, 7, 10] in epiphyseal cartilages of femoral bones.

The effect of pH on the arylsulphatase activity of acetone-dried distal and proximal cartilage and liver are presented in Fig. 3. The optimum pH for both cartilage enzymes was the same and corresponded to pH 7.5. The optimum for the liver enzyme was found to be at pH 7.8.

In Table 1 the results of experiments on the effect of different salts on enzymic activity are summarized. The activity of the samples to which no salt was added was taken as 1. The inhibiting effect of sulphite ions was very marked. Enzymic activity decreased to 0.8 in cartilage already at 10^{-4} M sulphite concentration while in the liver at 10^{-3} M. The inhibition by sodium fluoride was smaller. The decrease to 0.8 in the activity of both epiphyseal cartilages and the liver was observed at 10^{-2} M concentration.

Barium ions were added to incubation medium as chloride or acetate salts. No significant effect of barium chloride in two concentrations

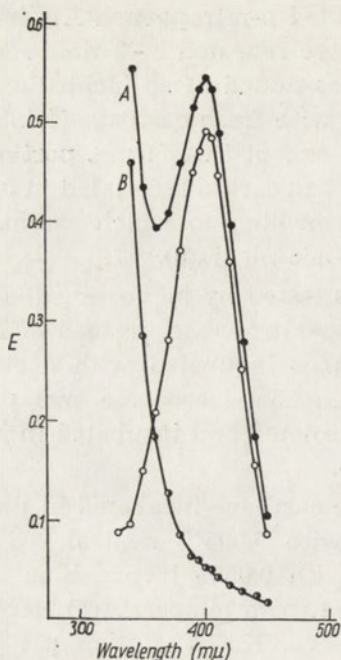


Fig. 2

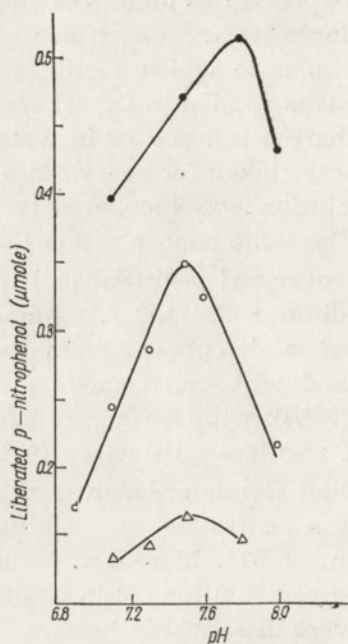


Fig. 3

Fig. 2. Liberation of *p*-nitrophenol from *p*-nitrophenyl sulphate by acetone-dried preparation of epiphyseal cartilage. Absorption spectra of samples incubated as described in methods. A (●), Proper sample; B (○), control, the cartilage preparation was added after incubation. The difference $A - B$ (○) shows the absorption spectrum characteristic for *p*-nitrophenol, absent in the medium at the beginning of incubation.

Fig. 3. The effect of pH on the arylsulphatase activity. Each sample contained 15 mg. of acetone-dried cartilage and 2.5 μ mole NPS in 1 ml. of 0.05M-tris-acetate buffer. Incubation: 1 hr. at 37°. Proximal epiphyseal cartilage of the femur (Δ), two experiments; distal cartilage (○), three experiments and for comparison liver (●), one experiment. Duplicate samples.

tested on the activity of cartilage enzyme was observed, while the activity of the liver enzyme increased to 1.4. Barium acetate, 0.025 M, slightly increased enzyme activity both in cartilages and in the liver. The effect of barium ions probably may be explained by the removal of the sulphate ions and shifting of the reaction to the right.

The activity of arylsulphatase C was determined in cartilage taken from different sites (Table 2). The activity of the distal epiphyseal cartilage of the femur was twice as high as that of the proximal one. Of the total activity of both epiphyseal cartilages, 2/3 was localized in the distal and only 1/3 in the proximal epiphysis. Enzymic activity in costal cartilage was lower than in epiphyseal cartilages.

Table 1

Effect of salts on arylsulphatase activity of acetone-dried cartilage

Each sample contained 15 mg. acetone-dried cartilage, 2.5 μ moles *p*-nitrophenyl sulphate and the respective salt as indicated in the Table, dissolved in 1 ml. of 0.05 M-tris-acetate buffer, pH 7.5. Incubation: 1 hr. at 37°. Duplicate samples; values from 1-3 experiments are shown. For comparison results on liver enzyme are included.

Addition	Concn. (mm)	Arylsulphatase activity		
		Epiphyseal cartilage		Liver*
		distal	proximal	
None	0	1.00	1.00	1.00
Na ₂ SO ₃	0.1	0.87	0.77	—
Na ₂ SO ₃	1	0.28, 0.27	—	0.89
NaF	10	0.80, 0.79	0.70, 0.88	0.82
BaCl ₂	25	0.97, 0.78, 0.99	0.92, 1.02	1.41
BaCl ₂	50	—	0.94	—
Ba(CH ₃ COO) ₂	25	1.10, 1.09	1.14	1.29
Mg(CH ₃ COO) ₂	50	1.17	1.11	1.31

* Data of B. Miks (in preparation).

Table 2

Arylsulphatase C activity in acetone-dried cartilage preparations

Each sample contained 15 mg. of acetone-dried cartilage, and 2.5 μ moles *p*-nitrophenyl sulphate in 1 ml. of 0.05 M-tris-acetate buffer, pH 7.5. Incubation: 1 hr. at 37°. Mean values, \pm standard error of the mean are given. The results are expressed as μ moles of *p*-nitrophenol liberated.

Cartilage	No. of expts.	<i>p</i> -Nitrophenol (μ moles)	%
Femur, epiphysis			
proximal	7	0.126 \pm 0.014	100
distal	7	0.253 \pm 0.035	205
Ribs	16	0.050 \pm 0.009	40

The fresh cartilage incubated with *p*-nitrophenyl sulphate also showed enzymic activity (Table 3). The amount of *p*-nitrophenol released by the fresh cartilage isolated and incubated in isotonic saccharose medium was lower (0.076 μ mole/hr.) than that released by cartilage isolated in water and incubated without saccharose (0.176 μ mole/hr.).

The survival of fresh cartilages was controlled by two tests: staining with neutral red and the permeability test for acidic dyes such as

Table 3

Arylsulphatase activity of fresh cartilage isolated and incubated in hypotonic or isotonic solutions

Each sample contained 50 mg. cartilage and 2.5 μ moles *p*-nitrophenyl sulphate in 1 ml. of 0.05 M-tris-acetate buffer, pH 7.5. The results are expressed as μ moles of liberated *p*-nitrophenol. The isotonic incubation medium contained 0.25 M-saccharose. The results obtained in survival tests (see text) are also included.

Incubation at 37° (min.)	Hypotonic medium	Isotonic medium
	(μmole <i>p</i> -nitrophenol)	
30	0.112	0.044
60	0.176	0.076
Survival tests:		
Neutral red, 1:20 000	—	+
Trypan blue, 1:2 000	+	—, +
Lissamine green, 1:1 000	+	—, +

trypan blue and lissamine green. In the fresh cartilage, as well before as after incubation in isotonic saccharose medium, most chondrocytes accumulated neutral red in vacuoles while the cytoplasm remained unstained. Chondrocytes showed the same staining reaction with neutral red when *p*-nitrophenol was added to 0.1 mM-concentration, that is to the concentration which may appear in the incubation medium as the result of the enzymic activity of the fresh cartilage. The results of staining with trypan blue and lissamine green appear to be more complicated. In the vicinity of the articular surface, the cells were stained with both stains before as well as after incubation. The cells lying further from the articular surface were not permeable for the stains. The above results of supravital staining permit to conclude that during the enzymic reaction in isotonic medium the cartilage cells do not change their staining properties and most probably remain alive, and that the living cartilage possesses enzymic activity.

As could be expected, the cells of fresh cartilage isolated in water and incubated without saccharose were permeable to trypan blue and lissamine green and did not accumulate neutral red in vacuoles.

DISCUSSION

Three types of arylsulphatases have been described [6, 11]; types A and B are soluble in water, type C is firmly bound to cellular structure and insoluble in water [7, 10].

The obtained results indicate the presence of arylsulphatase C in epiphyseal cartilage of rat femur. The optimal pH for the enzyme was similar to that for arylsulphatases C in rat [3] and human [5] liver. The enzyme was poorly soluble in water. The inhibitory effect of fluoride ions was but slight, similarly as that reported for enzymes from human [5] and bovine [10] liver.

The increase in length of the femoral bone in rat and man is due in about 2/3 to the growth of the distal epiphysis and in 1/3 to the growth of the proximal one [9]. It seems noteworthy that the activity of arylsulphatase C is distributed in a similar way, 2/3 being found in the distal and 1/3 in the proximal epiphyseal cartilage. As this may suggest physiological significance of arylsulphatase C in bone-growth processes, it would be interesting to compare the enzymic activity in both epiphyseal cartilages in birds, in which the bone is known to grow equally on both ends [9].

The author is indebted to Professor Dr. E. Kowalski as well as to Dr. B. Biczowa and Dr. W. Bicz for helpful discussions during the course of these experiments. This work was in part supported by the Department of Experimental Pathology of the Polish Academy of Sciences.

SUMMARY

The presence of arylsulphatase C activity was demonstrated in epiphyseal cartilages of the rat. The activity is higher in the distal than in the proximal epiphysis of the femur. Arylsulphatase activity was demonstrated also in the surviving cartilage.

REFERENCES

- [1] Burghardt G. N. & Lapworth A. - *J. Chem. Soc.* **1**, 684, 1926.
- [2] Dodgson K. S. & Spencer B. - *Biochem. J.* **53**, 444, 1953.
- [3] Dodgson K. S., Spencer B. & Thomas J. - *Biochem. J.* **53**, 452, 1953.
- [4] Dodgson K. S., Spencer B. & Thomas J. - *Biochem. J.* **59**, 29, 1955.
- [5] Dodgson K. S., Spencer B. & Wynn C. H. - *Biochem. J.* **62**, 500, 1956.
- [6] Dodgson K. S. & Spencer B., in *Methods of Biochem. Analysis*, (D. Glick, ed.) **4**, 211, Intersci. Publ., N. York, London, 1957.
- [7] Dodgson K. S., Rose F. A. & Spencer B. - *Biochem. J.* **66**, 357, 1957.
- [8] Dodgson K. S. & Tudball N. - *Biochem. J.* **74**, 154, 1960.
- [9] Heft J. - *Morfologie* **8**, 290, 1960.
- [10] Roy A. B. - *Biochem. J.* **64**, 651, 1956.
- [11] Roy A. B. - *Advanc. Enzymol.* **22**, 205, 1960.
- [12] Walkenstein S. S. & Knebel C. M. - *Analyt. Chem.* **29**, 1516, 1957.

AKTYWNOŚĆ ARYLSULFATAZY C W CHRZĄSTCE SZCZURA

Streszczenie

W chrząstkach nasadowych szczura wykazano obecność arylsulfatazy C. W nasadzie dalszej kości udowej aktywność jest wyższa niż w nasadzie bliższej. Aktywność arylsulfatazy stwierdzono również w chrząstce przeżywającej.

Received 29 June 1963

K. OSTROWSKI, Z. DARŻYNKIEWICZ, W. SAWICKI and ZOFIA STOCKA

**DRY MASS OF EPITHELIAL CELLS FROM THE VAGINA
OF MICE IN THE COURSE OF THE OESTRUS CYCLE MEASURED
BY INTERFERENCE MICROSCOPE**

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The vaginal epithelium undergoes considerable changes during the oestrus cycle which reflect changes in the function of ovaries and, as it is assumed by some authors, may be evoked by other hormones [16, 3].

The influence of the ovaries on the cytological and histological picture of vaginal epithelium is well known. The analysis of morphological changes in the vaginal smears, first introduced by Stockard & Papanicolaou [14] has become a valuable method for determining the functional condition of ovaries, and cytological examinations of vaginal smears are broadly applied in gynecology [3, 15].

In determining the hormonal function of ovaries, in normal and pathological conditions, the principal criterion is the degree of keratinization of the vaginal epithelium. The process of keratinization affects the superficial layers of epithelial cells, beginning with the condensing layer [13]. The cells of this layer and of the layers above exhibit various phases of keratinization while the superficial cells are completely corneous [1]. Continuous desquamation of the superficial cells is connected with proliferation of the epithelium and subsequent movement of the cells toward the lumen of the vagina. This movement is accompanied by morphological changes; the cells, at first small and cylindrical, become spherical, then large and multiangular and at last they are large, squamous and without nuclei.

According to the latest findings, keratinization is accompanied by extensive biochemical changes in the cells. These changes consist in the decomposition of proteins into amino acids and *de novo* synthesis of keratin [8, 5]. At the same time a great deal of water is lost and not more than 10% of water is present in corneous structures.

It may be assumed that these biochemical changes are associated with changes in the dry mass of cells. The purpose of this study was to determine the changes in the dry mass of cells that occur in the process of keratinization.

MATERIALS, METHODS AND APPARATUS

Preparation of smears. The experiment was carried out on smears of epithelial cells taken from the vagina of white mice of the C₅₇ strain. The mice were in the following three phases of the oestrus cycle: the dioestrus, prooestrus and oestrus. Consequently various quantities of cells at different stages of keratinization were obtained. The epithelial cells were classified into four types according to the consecutive changes occurring during keratinization (Fig. 1): Type I and II cells, oblong or

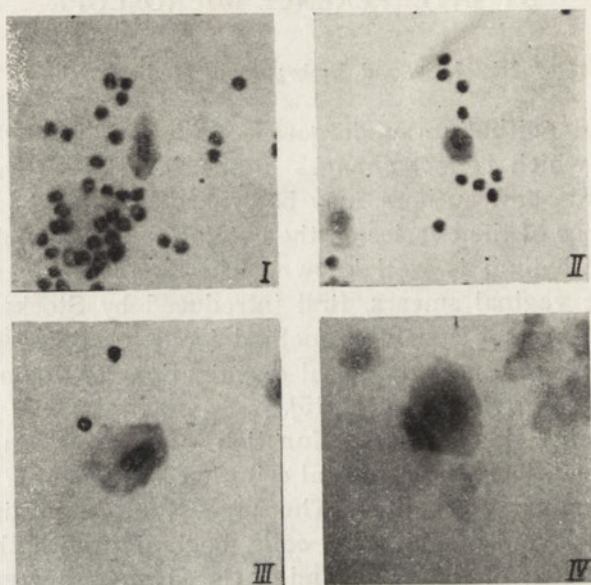


Fig. 1. Four types of epithelial cells of mouse vagina whose dry mass was determined.

spherical, and possessing large nuclei, were not keratinized; they were found in the germinal or lowest layer of the epithelium. Type III cells were much larger than type I and II; they were multiangular and their nuclei were in various stages of pycnosis. Finally, type IV cells were similar in shape and size to type III but had no nuclei. These last two types of cells were found in the layers of the epithelium nearest to the lumen of the vagina. Type I and II cells were found in the dioestrus phase, type III in the prooestrus and type IV cells in the oestrus. About 40 cells of each type were isolated and examined.

The cells were taken from the vagina and immediately suspended in Hanks solution of pH 7.4, and spread on slides. Each slide was covered with a coverglass, sealed with paraffin to prevent evaporation and

examined under an interference microscope. A part of smears was stained with hematoxylin & eosine.

Determination of dry mass. Davies *et al.* [6] and Barer & Joseph [4] stated that dry mass of a cell suspended in water may be expressed by the formula:

$$m = \frac{\Phi \cdot A}{100 a} \quad (1)$$

where m is the dry mass of cell expressed in grams; Φ , the optical path difference in relation to the medium; A , the surface of the measured structure in square centimeters; a , the specific refractive increment which is 0.0018 for biological structures (e.g. proteins, carbohydrates). If the cells are immersed in a medium other than water then the following correction to formula (1) should be introduced:

$$m = \frac{\Phi \cdot A}{100 a} + (\mu_m - \mu_w) \frac{A \cdot t}{100 a} \quad (2)$$

where $(\mu_m - \mu_w)$ is the difference in refractive index between the medium used and water, t , the thickness of the cell.

This formula could not be applied for calculating the dry mass of multiangular cells with a rough surface (type III and IV) since it was virtually impossible to determine their thickness (t). Type II cells, to simplify calculations, were considered as spheric, and the value of $(\mu_m - \mu_w) = 0.0016$ for Hanks solution [10] at 24° was adopted. The correction to formula (1) calculated in this way amounted to 1-2% of the dry mass. Consequently the correction for this difference could be omitted in view of high values of standard deviation for cell surfaces and measured path differences (see Table 1). A similar type of reasoning taking into account differences in the shape of cells, was adopted to the cells of the type I, III and IV.

The MPI interference microscope produced by Polskie Zakłady Optyczne, Warszawa, was employed for measuring the path differences. This microscope is based on the principle of interference of polarized light diffracted by a modified Wollaston prism. The prism serves both to split the light rays and to measure path differences between the light beam that passes through the object and the light beam that passes through the medium adjacent to the object. Depending on the type of double image prism used, the MPI interference microscope may be employed in three methods of quantitative studies: the interference shearing method, the wide double-image on a uniform field of view, and differential interference method [11, 12].

In the present study the wide double-image (about 15μ) in relation to the plane of the object on a uniform field was used. The measure-

ments were taken in monochromatic light (λ , 0.53 μ). The path difference was expressed by:

$$\Phi = \frac{p \cdot \lambda}{i} \quad (3)$$

where λ is the wavelength; i , the distance between the fringes of the double-image prism ($i = 770 \mu$); p , the transverse rotation of the prism corresponding to the difference between the positions in which first the background in the field of view and then one of the two images of the cell becomes dark.

The following measurements were made for each cell containing a nucleus: Φ_1 , path difference of the cytoplasm outside the nucleus; Φ_2 , path difference of the nucleus together with the cytoplasm over- and underlying the nucleus (Fig. 2). A third measurement, Φ_3 , path difference of the nucleus in relation to the cytoplasm, was made when the cell was so large that the double image of the nucleus did not reach beyond the coinciding areas of the cytoplasm images (Fig. 3), and consequently

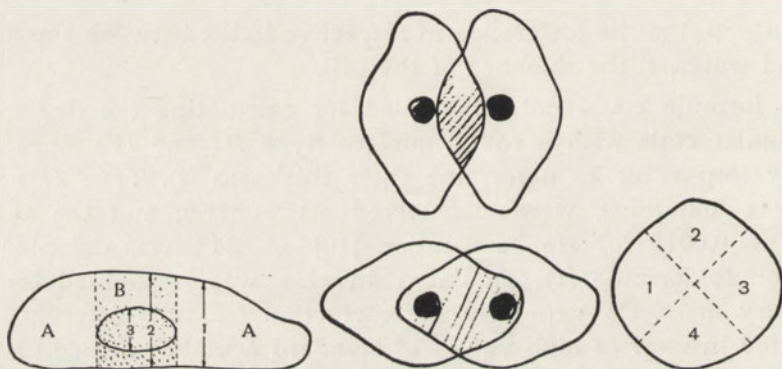


Fig. 2

Fig. 3

Fig. 4

Fig. 2. The scheme of the cross section of the measured cell showing the different areas which were separately measured.

Fig. 3. Positions of cell nuclei in two cases of overlapping of double images of the cell.

Fig. 4. The four sectors of the cell wherein the measurements were made.

it was impossible to measure Φ_2 ; therefore Φ_2 was obtained by adding Φ_1 and Φ_3 . The path differences were measured in each cell in four sectors (Fig. 4). Three measurements were made in each area and the average value was used for calculating the dry mass. Total dry mass of a nucleated cell was obtained by adding the dry mass values calculated separately from the Φ_1 path difference (Fig. 2, area A) and Φ_2 (Fig. 2, area B).

Planimetry of cell surfaces. Microphotographs were made of each cell before taking interferometric measurements. The microphotographs

were projected on filter paper, three projections being made for each cell. These were then cut out, weighed, and the average was calculated. Knowing the geometric magnification of the cell projection, the weight of the filter paper and average weight of the cell projection, the cell surface was calculated separately for the cytoplasm and nucleus. The methodical error resulting from the above described procedure was 1.44%.

RESULTS

Planimetric data indicate that the surface of type I and II cells is considerably smaller than the surface of type III and IV. The average cell areas for all four types of cells, with standard deviation values and variation coefficients, are given in Table 1 and Fig. 5; the values of path

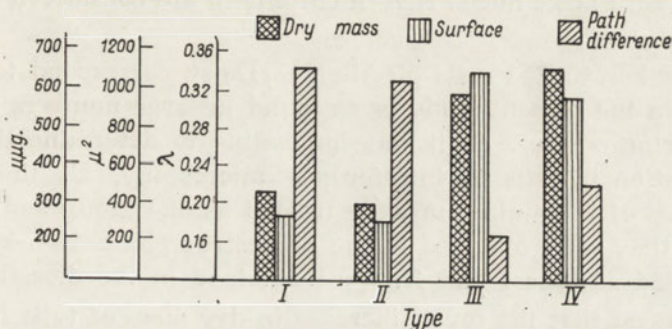


Fig. 5. Diagram illustrating the differences in dry mass, planimetry and optical path difference between the four types of epithelial cells.

differences and dry mass are also included. The average optical path difference is the highest for type I and II cells, while the average dry mass for type III and IV cells is more than double the value for types I and II. Standard deviation values and variation coefficients are high for all the obtained results.

Table 1

Dry mass, planimetry and optical path differences in four types of epithelial cells from mouse vagina in the course of keratinization

Mean values for one cell, \pm S. D., and variation coefficients (V) are given.

Cells	Dry mass		Surface		Optical path difference	
	($\mu\text{g.}$)	V(%)	(μ^2)	V(%)	(λ)	V(%)
Type I	313 \pm 113	36.0	331 \pm 141	42.6	0.35 \pm 0.09	25.7
Type II	273 \pm 81	29.7	283 \pm 118	41.6	0.34 \pm 0.11	32.3
Type III	626 \pm 280	44.7	1132 \pm 264	23.3	0.17 \pm 0.04	23.5
Type IV	655 \pm 290	44.0	1039 \pm 297	28.6	0.22 \pm 0.07	31.8

DISCUSSION

For evaluation of the keratinization process, the staining properties of cells have served so far as the principal criterion, while the shape of the cells and of their nuclei were rarely taken into account. For unstained cells, other methods of evaluation of keratinization had to be adopted and it was decided to employ the morphological criteria described by Allen [1] and Allen & Doisy [2]. These authors differentiated three types of epithelial cells from mouse vagina: (1), fully keratinized cells are large, multiangular, and without nuclei; (2), cells in various stages of keratinization are of the same size as fully keratinized cells but possess pycnotic nuclei; and (3), small spherical or oblong cells with large nuclei rich in chromatin are considered to be free of keratin.

The fully keratinized cells of Allen & Doisy correspond to type IV cells which in the present study were found in large numbers in smears obtained during oestrus. As it was impossible to determine the degree of keratinization in cells by interference microscopy, all the cells in various stages of keratinization were treated as one group and classified as type III; they were obtained in the prooestrus phase. Non-keratinized cells, classified as type I and II, were obtained in the dioestrus phase.

It is assumed that the found increase in dry mass of type III and IV cells as compared with type I and II is due to the formation and accumulation of keratin in the cells. It seems worth noting that there is only a small difference in dry mass between type III and type IV cells. If in type III cells the keratinization process still proceeds and only a small quantity of keratin has accumulated in the cytoplasm, then the difference in dry mass between these cells and the corneous type IV cells should be much greater. It seems that the large quantity of glycogen present in type III [7] which considerably increases the dry mass of these cells, may account for this effect.

Thus the process of keratinization which takes place when the epithelial cells move from the proliferative layer toward the lumen of the vagina, is connected with the increase of the dry mass and of the area of cells. Such quantitative descriptions of the development or differentiation of cells in the process of keratinization accompanied by qualitative and quantitative histochemical studies seem to hold promise for metabolic interpretation of intracellular changes.

The authors express their sincere gratitude to Mr. M. Pluta, M.Sc., the constructor of the interference microscope, for making the microscope available to the authors and for his valuable aid.

SUMMARY

Quantitative changes in dry mass and in the planimetry of epithelial cells from mouse vagina, in the process of keratinization were studied by interferometric measurements in the three phases of the oestrus cycle.

REFERENCES

- [1] Allen E. - *Am. J. Anat.* **30**, 297, 1922.
- [2] Allen E. & Doisy E. A. - *J. Am. Med. Assoc.* **81**, 819, 1923.
- [3] Allende I. N. C. & Orias O., *Cytology of Human Vagina*. P. B. Hoebel Inc. New York, 1950.
- [4] Barer R. & Joseph S. - *Quart. J. Microsc. Sci.* **95**, 399, 1954.
- [5] De Persaques J. & Rothman S. - *Nature* **193**, 147, 1962.
- [6] Davies H. G., Williams M. H. F., Chayen J. & La Cour L. F. - *Quart. J. Microsc. Sci.* **95**, 271, 1954.
- [7] Dierks K. - *Zentrblt. Gynäk.* **54**, 1882, 1930.
- [8] Herknes D. R. - *Acta Anat.* **31**, 35, 1957.
- [9] Kazanowska W. - *Ginekologia Polska* **31**, 451, 1960.
- [10] Ottoson R., Khan K. & Glick D. - *Exp. Cell Res.* **14**, 567, 1958.
- [11] Pluta M. - *Pomiary, Automatyka, Kontrola* (Warszawa) **7**, 183, 1961.
- [12] Pluta M. - *Pomiary, Automatyka, Kontrola* (Warszawa) **8**, 229, 1962.
- [13] Stemshern H. - *Zentrblt. Gynäk.* **52**, 2387, 1928.
- [14] Stockard C. & Papanicolaou C. N. - *Am. J. Anat.* **22**, 225, 1917.
- [15] Teter J., *Zaburzenia hormonalne u kobiety*. PZWL, Warszawa, 1956.
- [16] Zondek B., *Clinical and Experimental Investigation on the Genital Functions and their Hormonal Regulations*. Am. Soc. Clin. Invest., Baltimore, 1941.

INTERFEROMETRYCZNE OKREŚLANIE SUCHEJ MASY KOMÓREK NABŁONKA
POCHWY MYSZY W PRZEBIEGU CYKLU RUJOWEGO

Streszczenie

Posługując się mikroskopem interferencyjnym (MPI, produkcji PZO) określono zmiany suchej masy komórek nabłonka pochwy myszy w przebiegu cyklu rujowego. Wykazano znaczny wzrost suchej masy zrogowaciałych powierzchniowych komórek nabłonka pochwy w porównaniu z niezrogowaciałymi, podstawowymi komórkami.

Received 29 June 1963.

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A CONTRIBUTION TO THE STUDY OF GLUTATHIONE SYNTHESIS BY THE HUMAN ERYTHROCYTE

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The role of reduced glutathione (GSH) in the erythrocyte has been to a great extent explained. It has been shown that glutathione participates in metabolic processes which ensure the conversion of methaemoglobin to haemoglobin. Some enzymic chains of glycid metabolism are known in which glutathione plays an important role, and takes part in keeping the optimal shape of the erythrocyte and properties of its membrane. The permeability of the erythrocyte's cell membrane for sodium and potassium seems to depend to a considerable extent on the presence of free SH groups on its surface. The shape of the dissociation curve of oxyhaemoglobin is markedly influenced by the content of reduced glutathione in the erythrocyte, its increase causing the shifting of the curve to the right and its elongation, and *vice versa*.

Considerable attention has been paid to the biological significance of GSH for the erythrocyte and many efforts have been made to elucidate the pathways of glutathione synthesis and the mechanisms which ensure the stability of glutathione in the erythrocyte in its reduced form. The probable ways by which glutathione is introduced into the cell were also studied. It is known that this tripeptide does not penetrate from a medium into the erythrocyte; this was also confirmed by us in experiments in which the uptake both of cysteine and glutathione at 20° and 0° was followed. The results presented in Fig. 1 indicate that cysteine penetrates very rapidly into the erythrocytes while glutathione does not penetrate at all. Thus it is clear that the intra-erythrocyte GSH must be synthesized in the cell.

The synthesis of GSH was first studied on liver slices and on *Escherichia coli* cultures. The process has two stages; the first one consists in the formation of γ -glutamyl-cysteine in the presence of ATP; in the second, glycine is incorporated under the influence of glutathione synthetase. In the second stage the presence of ATP is also essential. Potassium stimulates this reaction [Crook, 1].

In the erythrocyte the synthesis of GSH proceeds very quickly. Miller & Horiuchi [7] have found that the incorporation of ^{14}C -labelled glycine as well as of ^{35}S -labelled cysteine occurs in 4 1/2 hr. It is of interest to note that the incorporation of [^{14}C]glutamic acid has not yet been demonstrated. In our experiments, using paper chromatography of extracellular medium and of haemolysate, glutamic acid did not penetrate almost at all into the erythrocyte. Glutamine, on the other hand, was

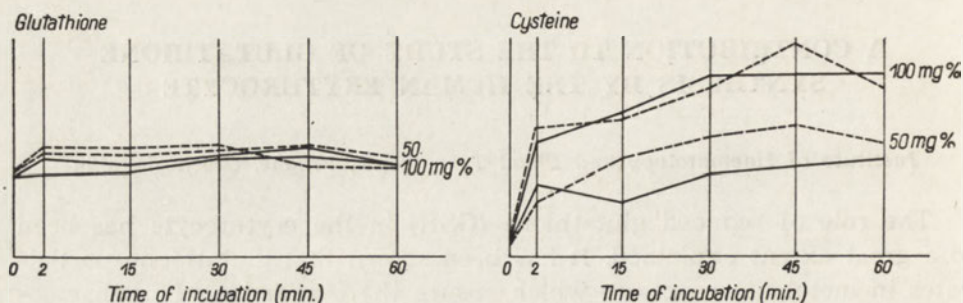


Fig. 1. The transfer of glutathione and cysteine from the medium into the human red cell. To the incubation medium containing the suspended erythrocytes, 50 or 100 mg. of GSH or cysteine were added (—), at 0° and (---), at 20°. At intervals indicated in the Figure the erythrocytes were isolated and haemolysed, and the concentration of the added compounds estimated.

found to penetrate and to be incorporated into glutathione [7]. According to Elder & Mortensen [3], the rate of GSH synthesis in the erythrocyte does not depend on the level of the endogenous glutathione; however, it decreases in the presence of oxygen as Koj [6] has also confirmed. Kasbekar & Sreenivasan [4] have found that Mg^{2+} and K^+ are necessary for the synthesis of GSH in the erythrocytes and that the addition of adenosine or adenosine-5'-monophosphate enhanced the synthesis.

Many experiments were carried out in which both the net increments of GSH and the incorporation of individual labelled amino acids were followed. On the basis of the results obtained in these experiments the synthesis of glutathione in the erythrocyte can be considered to be proved.

Our attention has been directed to two questions: (1), can GSH be synthesized only by the intact erythrocyte or also by the stroma alone? (2), can GSH be synthesized also from precursors other than the three constituent amino acids? That is, can cysteine be synthesized in the erythrocyte from methionine and serine, and can α -ketoglutaric acid replace glutamic acid? Recently Mirčevová *et al.* [8] reported that also in the erythrocyte this transamination occurs.

The reduced glutathione was estimated directly, using alloxan which forms a complex with GSH absorbing the ultraviolet light at the 305 m μ

region. Alloxan forms a similar complex with cysteine but the maximum absorption of this complex is at 275 μ so that no interference occurs. The modified method of Kay & Murfitt [5] (pH of the solution 7.6) gave very good results. The method was checked both by a calibration graph which showed a linear dependence in the range of 20 - 100 mg. % GSH, and by recovery tests.

Synthesis of GSH by erythrocyte stroma

Stroma was prepared from erythrocytes isolated from blood preserved with citrate and dextrose (ACD), by haemolysis with distilled water or ether. In haemolysis by water, 1 part of erythrocytes and 6 parts of water were used. Stroma isolated by centrifugation was washed twice with water. In haemolysis by ether, 1 part of erythrocytes was mixed with 0.1 part of ether; proteins were precipitated by saturation with ammonium sulphate and centrifuged off. The upper layer contained the stroma which was after separation washed once with physiological saline solution and twice with water.

Stroma, 0.5 ml., suspended in 3.5 ml. of Ringer-phosphate buffer, pH 7.2, was used for experiments. The medium contained also 0.025 M-glycine, 0.01 M-glutamic acid and 0.03 M-cysteine hydrochloride and 1.25 μ mole adenosine-5'-monophosphate. In one series of experiments 10 μ moles ATP was added. In addition to experiments with the direct GSH precursors, a medium was also used containing 0.03 M-methionine and 0.03 M-serine instead of cysteine, and 0.01 M- α -ketoglutaric acid instead of glutamic acid.

The results of the experiments showed that in freshly isolated, washed stroma an active synthesis of GSH took place if glycine, cysteine and glutamic acid were present in the incubation medium. On the other hand, when the medium contained serine and methionine, and α -ketoglutaric acid no increment of GSH could be observed after 3 hr. of incubation. The average increment of GSH in the Glu-Cys-Gly medium was about 210 μ moles (112, 200, 233, 297). No significant difference was observed if ATP was added to the medium.

Synthesis of GSH by intact erythrocytes

ACD erythrocytes isolated from the blood withdrawn on the same day or only one day old were used. The composition of the incubation media (Glu, Cys, Gly; α -ketoglut., Gly, Met, Ser) was the same as for the first series of experiments, except that the addition of adenyl-5'-monophosphate was 5 μ moles. The erythrocytes were incubated anaerobically in the appropriate medium for 3 hr. at 37°, nitrogen being passed through during the first 30 min. of incubation. These conditions were

strictly observed as it is known that the synthesis of GSH in erythrocytes is more active in the absence of oxygen.

The results presented in Table 1 indicate that glutathione can be synthesized in the erythrocytes not only from the three direct precursors, but also when serine and methionine are present in the medium instead of cysteine, and α -ketoglutaric acid instead of glutamic acid. No increment of GSH synthesis was observed when the haemolysate was used instead of the whole erythrocytes.

Table 1

Synthesis of glutathione by human erythrocytes

The results presented are averages; the limit values are given in parentheses.

Addition	Increment after 3 hr. incubation (μ moles)
None	29 (5.4 – 68)
Glu, Cys, Gly	297 (209 – 579)
Glu, Gly, Met, Ser	220 (164 – 328)
α -Ketoglutarate, Gly, Met, Ser	98 (91 – 101)

In two tests the total amount of GSH was determined after the electrolytic reduction of GSSG according to Dohan & Woodward [2]. The results of one of these experiments are shown in Table 2; the second one, evaluated only semiquantitatively, showed similar differences in GSH synthesis.

Table 2

Synthesis of total and reduced glutathione by human erythrocytes

Addition	Increment after 3 hr. incubation	
	GSH	Total GSH
	(μ moles)	
None	14	84
Glu, Cys, Gly	209	980
Glu, Gly, Met, Ser	164	250
α -Ketoglutarate, Gly, Met, Ser	101	339

The presented results show that the intact erythrocyte is able to synthesize glutathione not only from the direct precursors but also from α -ketoglutaric acid, methionine and serine.

Recently in our laboratory Mirčevová *et al.* [8] have succeeded in proving that in erythrocytes α -ketoglutaric acid can be transaminated

to glutamic acid. Since the penetration of glutamic acid from the medium into the erythrocyte proceeds very slowly it seems possible that in the erythrocyte the transamination reaction is used as the source of glutamic acid.

The synthesis of GSH in the presence of methionine and serine instead of cysteine indicates that in the erythrocyte the synthesis of cysteine proceeds by the generally known pathway.

Unlike the erythrocytes, stroma alone can form GSH only from the direct precursors. Probably the processes of transsulphuration and transamination require the presence of soluble enzymes which are removed during haemolysis and washing of the stroma. Therefore the present experiments should be completed by tests using labelled amino acids to follow the incorporation of individual components into glutathione. This will be the subject of our further study.

SUMMARY

1. In human erythrocytes the synthesis of glutathione from the direct precursors under anaerobic conditions in the presence of ATP, was confirmed. Stroma alone possesses the same ability.

2. Intact erythrocytes synthesize glutathione not only from the direct precursors but also when α -ketoglutaric acid is present instead of glutamic acid, and methionine and serine instead of cysteine. Stroma isolated and washed cannot synthesize GSH from the indirect precursors.

REFERENCES

- [1] Crook E. M., *Glutathione. Symposium of Biochemical Society*, no. 17, 1958.
- [2] Dohan J. S. & Woodward G. E. - *J. Biol. Chem.* **129**, 393, 1939.
- [3] Elder H. A. & Mortensen R. A. - *J. Biol. Chem.* **218**, 261, 1956.
- [4] Kasbekar D. K. & Sreenivasan A. - *Biochem. J.* **72**, 389, 1959.
- [5] Kay W. W. & Murfitt K. C. - *Biochem. J.* **74**, 203, 1960.
- [6] Koj A. - *Acta Biochim. Polon.* **9**, 11, 1962.
- [7] Miller A. & Horiuchi M. - *J. Lab. Clin. Med.* **60**, 756, 1962.
- [8] Mirčevová L., Jirgl V. & Vosyková J. - *J. Lab. Clin. Med.* **60**, 110, 1962.

PRZYCZYNEK DO BADAŃ NAD SYNTEZĄ GLUTATIONU W LUDZKICH ERYTROCYTACH

Streszczenie

1. W warunkach beztlenowych w obecności ATP potwierdzono w erytrocytach syntezę glutationu z jego bezpośrednich prekursorów. Izolowana stroma również syntetyzuje glutation.

2. Erytrocyty syntetyzowały glutation również wtedy, gdy zamiast kwasu glutaminowego dodano kwas α -ketoglutarowy, a zamiast cysteiny metioninę i serynę. Izolowana stroma w tych warunkach nie syntetyzowała glutationu.

Received 5 September 1963.

to obtain the same result as the one obtained in the case of the first two cases. It is seen from the above that in the case of the first two cases the result is the same as the one obtained in the case of the first two cases.

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CONCLUSION

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REFERENCES

1. J. K. P. (1980) The results of the first two cases are the same as the one obtained in the case of the first two cases.
2. J. K. P. (1980) The results of the first two cases are the same as the one obtained in the case of the first two cases.
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APPENDIX

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ZOFIA POREMBSKA and IRENA MOCHNACKA

THE ORNITHINE CYCLE IN *CELERIO EUPHORBIAE*

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In the majority of insects, uric acid is excreted as the end product of protein metabolism, and only the aquatic insects excrete most of the nitrogen as ammonia [17, 18]. The presence of urea in excreta of many insects has also been reported [for ref. see 5, 17] but the excretion of urea as the end product of protein metabolism in insects has often been disputed. Heller [6] has shown that it could arise from bacterial decomposition of uric acid in the hindgut, and suspected that urea found in some reported cases, especially when larger amounts of material were used for analysis, might have resulted from an infection. In some insects the urea found in excreta may represent the unchanged urea taken in the food. This is certainly so in *Rhodonius prolixus* as demonstrated by Wigglesworth [22]; the same can be true for the clothes moth in whose excreta large amounts of urea were found by Powning [13].

Ornithine and citrulline known as intermediates in urea biosynthesis have also been found in haemolymph and tissues of several insects [20, 11]. Kilby & Neville [8] found arginase activity in tissues of *Locusta migratoria*, and Szarkowska & Porembska [19] in tissues of *Celerio euphorbiae* at every stage of development. Carcia, Tixier & Roche [4] assumed recently that the pathway of urea formation in the bee, silkworm and locust is the same as in vertebrates.

It appears, however, that the metabolic role of urea in insects is not yet unequivocally established. In the present work the possibility of urea biosynthesis by way of the ornithine cycle in *Celerio euphorbiae* has been investigated.

MATERIAL AND METHODS

Caterpillars, pupae and adult moths of *C. euphorbiae* were studied. For the experiments extracts from acetone powders or from fresh whole animals as well as from isolated tissues were used. The extracts were prepared according to Brown & Cohen [1]. Fresh tissues or acetone powders were ground with 0.1% solution of cetyl trimethylammonium bromide (CTB) in all-glass Potter homogenizer at room temperature,

5 ml. of CTB being applied per 0.5 g. of fresh tissue, and 10 ml. of CTB per 0.5 g. of acetone powder. The suspension was chilled on ice and centrifuged for 10 min. at 5000 *g* at 0 - 2°.

Analytical procedure. Protein was determined by the Mejbaum-Ka-zenellenbogen tannin method [10]. Citrulline was assayed with dimethylglyoxime by the Fearon method as modified by Lazariew [9], urea by the xanthidrol method of Fosse as modified by Engel & Engel [3], and ammonia by the Conway's procedure using the Nessler reagent and colorimetric estimation [2]. The arginine content was calculated from the amount of urea formed upon the addition of arginase, which had been obtained from beef liver after Ratner [14]. The preparation of arginase contained 8 mg. of protein per 1 ml. and was diluted as required. For chromatographic examination of amino acids the fresh tissues were extracted with 60 - 70% ethanol, and two-dimensional descending technique on Whatman no. 1 paper in propan-1-ol - water (7:3, v/v) in the atmosphere of 0.3% ammonia was employed. The spots were located with 2% solution of ninhydrin in acetone.

Enzyme assays. The activity of ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine carbamoyltransferase EC 2.1.3.3) was examined in the system containing in 2 ml.: 50 μ moles of glycine buffer, pH 8.5, 20 μ moles of carbamoylphosphate, 20 μ moles of L-ornithine, and enzyme extract. The incubation was carried out for 30 min. at 25°. The reaction was stopped by adding 2 ml. of 1 N-perchloric acid; after centrifugation citrulline was estimated in the supernatant.

The synthesis of arginine from citrulline and aspartate was assayed in the incubation mixture containing in 1.2 ml.: 50 μ moles of tris buffer, pH 7.2, 5 μ moles of L-citrulline, 5 μ moles of ATP, 5 μ moles of aspartate, 5 μ moles of MgSO₄, arginase solution and enzyme extract. The incubation was carried out for 1 hr. at 25°, and the reaction stopped by adding 1.5 ml. of 83% acetic acid. The sediment was discarded and in the supernatant the amount of urea (formed by the added arginase) was determined.

The activity of arginase was examined in the mixture containing in 1 ml.: 50 μ moles of glycine buffer, pH 9.5, 0.5 μ moles of MnCl₂, 25 μ moles of arginine, and enzyme extract. Samples were incubated for 30 min. at 25°. The reaction was stopped by adding 1.5 ml. of 83% acetic acid. After centrifugation urea was determined in the supernatant.

Special reagents. L-Citrulline hydrochloride and L-arginine hydrochloride (L. Light, England); L-ornithine hydrochloride and L-aspartic acid (Riedel, West Germany); ATP (Schwarz, Mount Vernon, U.S.A.); cetyl trimethylammonium bromide (British Drug Houses, Ltd.); carbamoylphosphate dilithium salt of 65 - 75% purity was prepared from potassium cyanate and potassium dihydrogen phosphate after Jones, Spec-
tor & Lipmann [7].

RESULTS AND DISCUSSION

To study the enzymes of the ornithine cycle the extracts were prepared with 0.1% solution of CTB, which according to Brown & Cohen [1] is a good extractant of these enzymes. No synthesis of citrulline from ornithine and carbamoylphosphate was observed in the fresh or acetone-dried tissues of *C. euphorbiae* at any stage of development. In Table 1 the results of experiment with extract from acetone-dried fat-body and muscles of the adult moth are given. Small amounts of citrulline formed during 30 min. incubation can be accounted for as resulting from non-enzymic synthesis, since they were also observed in samples without the extract added. For the non-enzymic synthesis of citrulline or of other compounds reacting with dimethylglyoxime the presence of carbamoylphosphate appeared to be essential. This is in agreement with the non-enzymic formation of citrulline and of other ornithine derivatives demonstrated by Reuter [16] and Reifer & Kleczkowski [15]. Under the conditions of our experiments, the rate of non-enzymic synthesis was low; it could be increased significantly by prolonging the time of incubation to several hours and by raising the temperature to about 40°.

Table 1

Citrulline synthesis in extracts from acetone-dried fat-body and muscles of the adult Celerio euphorbiae moth, and ox liver

Incubation mixture contained in 2 ml.: 50 μ moles of glycine buffer, pH 8.5, 20 μ moles of ornithine, 20 μ moles of carbamoylphosphate, and CTB extract from acetone-dried tissues or from ox liver (1.7 mg.-0.2 mg. of protein, resp.). Incubation: 30 min. at 25°. Average values from 6 experiments are given, in parentheses the limit values.

Sample	Citrulline formed (μ moles)
<i>C. euphorbiae</i>	
Whole system	0.04 (0.02 – 0.05)
Ornithine omitted	0.03 (0.02 – 0.04)
Carbamoylphosphate omitted	0.0
Extract omitted	0.04 (0.02 – 0.04)
Ox liver	
Whole system	0.81 (0.77 – 0.9)
Whole system and extract from <i>C. euphorbiae</i> tissues	0.82 (0.71 – 0.89)

The possibility of an inhibitor of ornithine carbamoyltransferase being present in the tissues of *C. euphorbiae* has been excluded, as the addition of the tissue extract to the extract from ox liver had no effect on the rate of citrulline synthesis (Table 1).

Despite the lack of citrulline synthesis in the caterpillar, pupa and adult moth, citrulline is present in their tissues as revealed by paper chromatography (Fig. 1 a, b). It might have been derived from the ingested food, as its presence was also demonstrated in the *Euphorbia* plant (Fig. 1 c). However, this does not exclude the possibility of endogenous formation of citrulline by a pathway other than the ornithine cycle e.g.

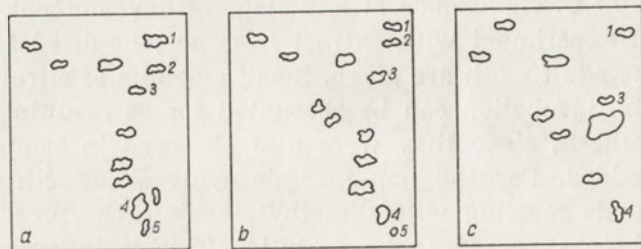


Fig. 1. Paper chromatograms of 70% ethanol extracts of *Celerio euphorbiae* (a) muscle, (b) fat-body, and (c) of *Euphorbia* plant. (1), Arginine; (2), ornithine; (3), citrulline; (4), glutamic acid; (5), aspartic acid.

the degradation of arginine by arginine iminohydrolase, which was found in bacteria [12]. The remaining amino acids of the ornithine cycle i.e. ornithine, arginine and glutamic and aspartic acids have also been found in tissues of *C. euphorbiae* by chromatographic analysis (Fig. 1 a, b).

The enzymic formation of arginine from citrulline and aspartate in the presence of ATP was demonstrated in extracts of fresh and acetone-dried tissues from *C. euphorbiae* at different stages of development. Table 2 represents the results of an experiment with adult moth tissues. In contrast to ox liver, the addition of citrulline and aspartate was not necessary, although when no citrulline and/or aspartate was added, the synthesis was lower by 20–30%, and by 70% without the addition of ATP. As non-dialysed extracts were used for the experiment, it could be assumed that when no citrulline and aspartate were added, the own endogenous substrates would be used for the synthesis. Therefore the experiments were repeated with dialysed extracts. As the dialysis of extracts from ox liver did not cause an inactivation of the enzymic system, the same was expected to be true for *C. euphorbiae*. However, the dialysed extracts from moth tissues proved to be inactive. These results may lead to the supposition that arginine synthesis in *C. euphorbiae* may proceed through a different pathway.

The presence of arginase in the tissues of *C. euphorbiae* demonstrated previously in water homogenate [19] now has been confirmed with CTB-extracts from acetone-dried tissues. No urease was detected in the tissues of *C. euphorbiae*.

The presented experiments failed to demonstrate the formation of

Table 2

Arginine synthesis in extracts from fresh muscle and fat-body of the adult moth Celerio euphorbiae and in extract from acetone-dried ox liver

Incubation mixture contained in 1.2 ml.: 50 μ moles of tris buffer, pH 7.2, 5 μ moles of citrulline, 5 μ moles of aspartate, 5 μ moles of ATP, 5 μ moles of $MgSO_4$, 0.2 ml. of arginase solution (0.3mg. of protein) and CTB extract from *C. euphorbiae* or from ox liver (4 and 2mg. of protein, resp.). Incubation: 1 hr. at 25°. Arginine was measured as the amount of urea formed upon the addition of arginase. Urea was determined by the xanthidrol method. Average values from 6 experiments are given, in parentheses the limit values.

Sample	Arginine formed (μ moles/sample)	
	<i>C. euphorbiae</i>	Ox liver
Whole system	0.64 (0.4 – 0.7)	1.01 (1.0 – 1.17)
Citrulline omitted	0.4 (0.31 – 0.45)	0.1 (0.07 – 0.12)
Aspartate omitted	0.47 (0.3 – 0.5)	0.09 (0.05 – 0.14)
ATP omitted	0.2 (0.0 – 0.35)	0.03 (0.02 – 0.036)
Citrulline and ATP omitted	0.23 (0.1 – 0.35)	—
Aspartate and ATP omitted	0.21 (0.13 – 0.34)	—
Citrulline and aspartate omitted	0.49 (0.21 – 0.58)	—
Boiled extract	0.03 (0.02 – 0.05)	0.04 (0.03 – 0.043)
Extract omitted	0.0	0.0

urea in *C. euphorbiae* by the way of the ornithine cycle. Moreover, the synthesis of carbamoylphosphate has not been demonstrated in insects. The study of Garcia, Tixier & Roche [4] indicated the conversion of arginine into citrulline, and the relationship between ornithine, citrulline and arginine; these results were interpreted by the authors in favour of the occurrence of the ornithine cycle in insects but this evidence is not unequivocal.

The arginase found in insects need not be related to the biosynthesis of urea. In *C. euphorbiae* it could possibly catalyse the cleavage of arginine which is present there in large amounts in the form of phosphoarginine.

The way of arginine synthesis in *C. euphorbiae* is not clear. Since dialysis inactivates the formation of arginine from citrulline and aspartate, another pathway of arginine synthesis should be considered. It is worth remembering that Szörenyi, Elödi & Pusztai [21] found that in the crayfish phosphoarginine is synthesized from citrulline, ammonia and ATP in the presence of pyridoxal phosphate.

SUMMARY

Enzyme activity was assayed in tissue extracts made with 0.1% solution of cetyl trimethyl ammonium bromide. No citrulline synthesis from ornithine and carbamoylphosphate was observed. Synthesis of arginine was ATP-dependent but when no citrulline and aspartate were added, the synthesis decreased only by 30%. The presence of arginase was confirmed. By paper chromatography, ornithine, citrulline, arginine and glutamic and aspartic acids were found to be present in *C. euphorbiae* tissues.

REFERENCES

- [1] Brown G. W. Jr. & Cohen P. P. - *J. Biol. Chem.* **234**, 1769, 1959.
- [2] Conway E. J., *Microdiffusion Analysis and Volumetric Error*, Lockwood, London 1947.
- [3] Engel M. G. & Engel F. L. - *J. Biol. Chem.* **167**, 535, 1947.
- [4] Garcia I., Tixier M. & Roche J. - *C. R. Soc. Biol.* **150**, 632, 1956.
- [5] Gilmour D., *Biochemistry of Insects*, p. 178. Acad. Press, New York and London 1961.
- [6] Heller J. & Aremówna H. - *Z. vergl. Physiol.* **16**, 362, 1932.
- [7] Jones M. E., Spector L. & Lipmann F. - *J. Am. Chem. Soc.* **77**, 819, 1955.
- [8] Kilby B. A. & Neville E. J. - *J. Exptl. Biol.* **34**, 276, 1957.
- [9] Łazarjew A. F. - *Biochimia* **15**, 401, 1950.
- [10] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **2**, 279, 1955.
- [11] Pant R. & Agrawal H. C. - *Arch. Intern. Physiol. Biochim.* **71**, 605, 1963.
- [12] Petrack B., Sullivan L. & Ratner S. - *Arch. Biochem. Biophys.* **69**, 186, 1957.
- [13] Powning R. F. - *Australian J. Biol. Sci.* **6**, 109, 1953.
- [14] Ratner S. in *Methods in Enzymology*, Vol. II, p. 357 (Eds. S. P. Colowick & N. O. Kaplan) Acad. Press, New York 1955.
- [15] Reifer I. & Kleczkowski K. - *Z. Naturforschung* **15b**, 431, 1960.
- [16] Reuter G. - *Z. Naturforschung* **14b**, 475, 1959.
- [17] Roeder K. D., *Insect Physiology*, pp. 176, 396. John Wiley, New York, Chapman Hall, London 1953.
- [18] Staddon B. W. - *J. Exptl. Biol.* **32**, 84, 1955.
- [19] Szarkowska L. & Porembska Z. - *Acta Biochim. Polon.* **6**, 273, 1959.
- [20] Szörenyi E. T., Elödi P. & Deutsch T. - *Acta Physiol. Acad. Sci. Hung.* **5**, 337, 1954.
- [21] Szörenyi E. T., Elödi P. & Pusztai A. - *Acta Physiol. Acad. Sci. Hung.* **7**, 163, 1955.
- [22] Wigglesworth V. B., *The Principles of Insect Physiology*, 4th ed. Methuen, London 1950.

CYKL ORNITYNOWY U *CELERIO EUPHORBIAE*

Streszczenie

Aktywność enzymów badano w wyciągach z tkanek ekstrahowanych 0.1% roztworem bromku cetylotrójmetylo amonowego. Nie stwierdzono syntezy cytruliny z ornityny i karbamylfosforanu. Synteza argininy zależna była od ATP, nie dodanie cytruliny i kwasu asparaginowego zmniejszało syntezę jedynie o 30%. Potwierdzono wykazaną w poprzedniej pracy arginazę. Chromatografią bibułową stwierdzono w tkankach *C. euphorbiae* obecność ornityny, cytruliny, argininy oraz kwasu glutaminowego i kwasu asparaginowego.

Received 3 October 1963.

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SERUM GLYCOPROTEINS IN SOME SPECIES OF NON-DOMESTICATED MAMMALS

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The results of our previous studies [3] seemed to indicate that in the serum of mammals the electrophoretical patterns of glycoproteins and the content of hexoses bound with total protein and with mucoproteins are species-dependent. In the present work similar estimations as well as sialic acid determinations were performed on eight species of non-domesticated animals.

MATERIALS AND METHODS

Blood samples were obtained from the buffalo, european bison, lion, tiger, fallow deer, red deer, and brown bear by puncture of the jugular vein, the animals being anaesthetized by eunarcon, morphine, chloral hydrate or narcosan. From european (brown) hare the blood was taken from the heart without narcosis.

Paper electrophoresis. Under the conditions described previously [3] the electrophoretical separation of serum glycoproteins was not wholly satisfactory when the level of protein was low; depending on the amount of serum applied, the fractions were either too feebly stained or the separation was not quite satisfactory. The procedure described below seems to assure good separation of serum glycoproteins even when there are large variations in serum protein content.

Electrophoresis was carried out at room temperature in the apparatus described by Wieland & Fisher [10] on Whatman no. 1 paper strips 3.5 cm. wide. The buffer used was the medinal-veronal solution, pH 9.0, I 0.1, potential 80 V, and time of electrophoresis 18 hr. For assays of protein fractions 7-10 μ l. of serum was applied, and for glycoprotein 50-60 μ l. For comparison of electrophoretic patterns a sample of human serum was simultaneously separated. Human serum was obtained from one donor and was kept frozen in small portions suitable for single determinations.

The electrophoregrams were stained with Amido Black for proteins and with Schiff's reagent for glycoproteins as described previously [3]. Densitometric curves were obtained in a Zeiss apparatus, type E.R.J.10. The mobility of fractions was calculated according to de Wael & Punt [8].

Chemical analysis and paper partition chromatography. Protein was determined by the biuret method [9]. Hexoses bound with total serum protein or with seromucoid¹, and sialic acid were estimated by the methods of Dżułyńska & Piekarska [1, 2]. As the presence of neuraminic acid derivatives differing in molar absorption was anticipated, these compounds were isolated from total serum protein by the method of Svennerholm [7] and subjected to paper chromatography according to Martensson *et al.* [5]. *N*-Glycolylneuraminic acid kindly given by Dr. G. Uhlenbruck from the Institute of Physiological Chemistry in Köln (West Germany), and *N*-acetylneuraminic acid prepared according to the method of Svennerholm [7] were used as standards. The content of *N*-glycolylneuraminic acid in the obtained preparations was assayed by the method of Klenk & Uhlenbruck [4] in the modification of Martensson *et al.* [5].

RESULTS

The results of electrophoretical separation of serum glycoproteins are presented in Table 1. Glycoprotein fractions in different species differed in electrophoretical mobility, quantitative distribution and even in number. But within the species the protein fractions and the fractions stained with Schiff's reagent were similar in mobility and number. Only in the hare instead of two adjacent protein fractions β' and γ , a single glycoprotein fraction was found with a mobility intermediate between that of β' - and γ -globulins (see Fig. 1).

Similarly as in the previous studies [3] the largest amount of substances staining with Schiff's reagent was found in α -globulin fractions. Only in the bear and red deer sera a very high glycoprotein content was found in β -globulins.

To permit comparison of glycoprotein fractions within a taxonomic family, estimations were also performed on blood sera of domesticated animals related to the examined species of non-domesticated animals. The densitometric curves obtained (Fig. 2) showed that the electrophoretical patterns of glycoprotein fractions are very similar for Bovidae (cattle and bison) and Felidae (cat, lion and tiger) but dissimilar for Cervidae (fallow deer and red deer) and Leporidae (rabbit and hare).

Serum glycoproteins were further tested by chemical methods for the content of hexoses and sialic acid bound with total serum protein

¹ The term "seromucoid" instead of "mucoprotein" used in the previous work, has been applied in agreement with the recently adopted nomenclature [11].

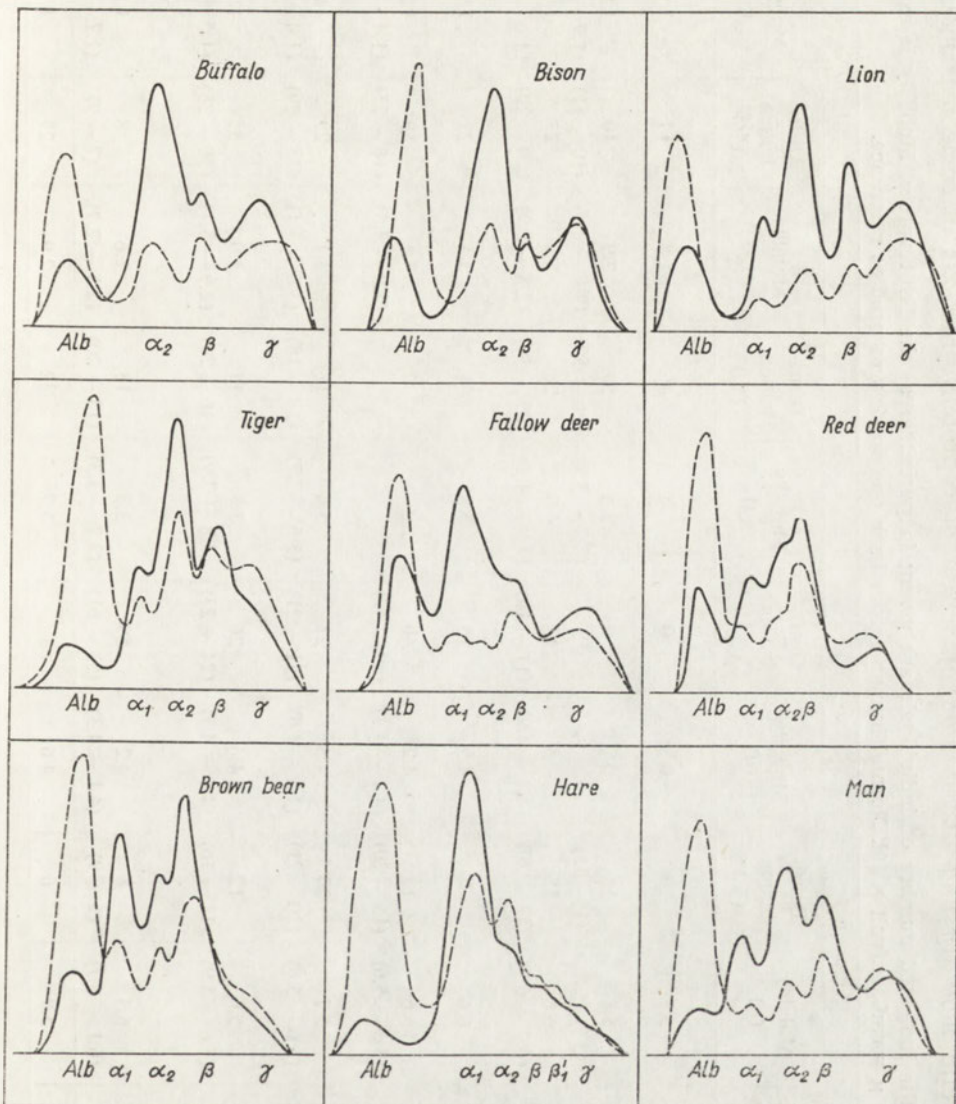


Fig. 1. Densitometric curves of serum glycoprotein fractions of some non-domesticated mammals. Human serum is included for comparison. (---), Proteins; (—), glycoproteins.

Table 1

Electrophoretic mobility and distribution of serum glycoprotein fractions in some mammals

Average values for the indicated number of individuals are given; in parentheses the limit values. Mobility is expressed as $\mu = \text{cm}^2/\text{sec.}/\text{volt} \times 10^5$. Normal human serum from one donor was used as reference.

Species	No. of individuals	Albumin		α_1		α_2		β		γ	
		Mobility (μ)	Distribution (%)	Mobility (μ)	Distribution (%)	Mobility (μ)	Distribution (%)	Mobility (μ)	Distribution (%)	Mobility (μ)	Distribution (%)
Buffalo, <i>Bubalus bubalis</i>	1	5.7	20	0	0	3.8	46	2.8	14	1.5	20
European bison, <i>Bison bonasus</i>	3	5.8 (5.7-5.8)	15 (15-15)	0	0	3.8 (3.7-3.9)	50 (50-50)	2.9 (2.8-3.0)	10 (10-11)	1.8 (1.7-1.9)	25 (24-25)
Lion, <i>Panthera leo</i>	3	6.8 (6.7-6.9)	18 (14-20)	4.8 (4.7-4.9)	13 (11-16)	4.0 (3.9-4.1)	28 (27-30)	2.9 (2.8-2.9)	23 (20-29)	1.7 (1.6-1.8)	18 (14-22)
Tiger, <i>Panthera tigris</i>	1	6.6	8	4.9	15	3.9	41	3.1	22	1.9	14
Fallow deer, <i>Dama dama</i>	5	5.6 (5.5-5.6)	18 (15-20)	4.2* (4.1-4.3)	40 (39-42)	0	0	3.1 (3.0-3.3)	19 (18-23)	1.5 (1.4-1.7)	23 (20-26)
Red deer, <i>Cervus elaphus</i>	2	5.7 (5.6-5.8)	20 (20-21)	4.5 (4.5-4.6)	21 (21-21)	3.6 (3.6-3.7)	18 (17-18)	3.1 (3.0-3.1)	29 (28-29)	1.6 (1.4-1.7)	12 (12-13)
Brown bear, <i>Ursus arctos</i>	4	5.6 (5.6-5.9)	12 (11-16)	4.6 (4.6-4.7)	26 (24-27)	3.8 (3.8-3.9)	17 (16-20)	3.0 (2.9-3.1)	35 (30-38)	1.7 (1.5-2.0)	10 (9-11)
European hare, <i>Lepus europaeus</i> Pallas	4	6.3 (6.1-6.7)	8 (5-9)	4.2 (4.1-4.3)	59 (53-61)	3.3 (3.2-3.3)	18 (13-20)	2.6 (2.5-2.7)	8 (7-9)	1.8** (1.7-1.8)	7 (7-8)
Man	1	5.9	6	4.6	17	3.7	32	2.9	27	1.5	18

* Glycoprotein fraction with mobility intermediate between that for α_1 - and α_2 -protein fractions.

** Glycoprotein fraction with mobility intermediate between that for β '- and γ -protein fractions (see Fig. 1).

and seromuroid (Table 2). From serum proteins of buffalo, bison, lion, tiger, fallow deer and hare, sialic acid was isolated and identified by paper partition chromatography. On the chromatograms only two spots of neuraminic acid derivatives were found; their R_F values corresponded to those of the parallelly applied standards of *N*-acetylneuraminic and *N*-glycolylneuraminic acids. As the amounts of blood obtained from the bear and red deer were insufficient, sialic acid was not isolated.

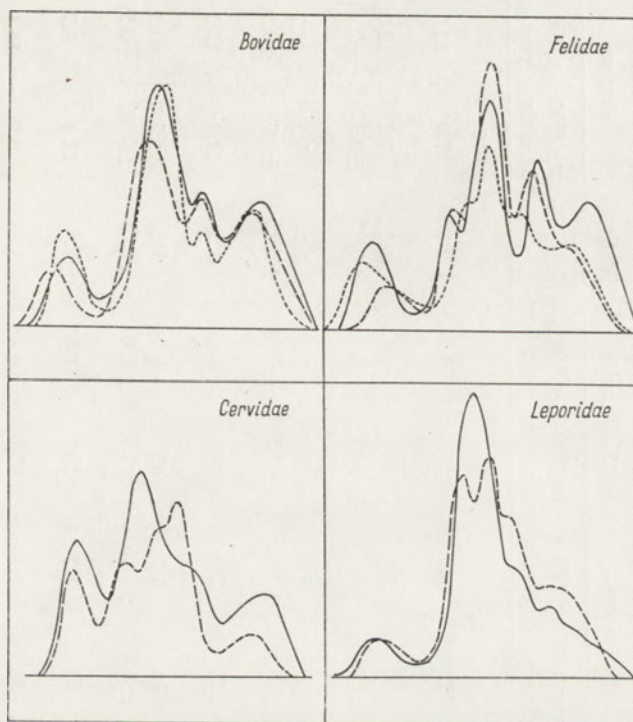


Fig. 2. Comparison of densitometric curves for related species of mammals. Bovidae: (—), cattle; (---), European bison; (- - -), buffalo, Felidae: (—), lion; (---), tiger; (- - -), cat. Cervidae: (—), fallow deer; (---), red deer. Leporidae: (—), European hare; (---), rabbit.

Marked differences in the ratio of *N*-acetylneuraminic acid to *N*-glycolylneuraminic acid were observed in total serum protein of animals belonging to different families. Differences were also observed between the animal species in the content of hexoses and sialic acid bound with total protein and seromuroid in 100 ml. of serum, as well as in the content of these carbohydrates in 100 g. of proteins. The ratio of hexoses to sialic acid in animals belonging to different families showed large differences which were most marked in seromuroids. On the other hand, for animals belonging to the same family the values obtained were very

Table 2

Protein-bound hexoses in serum of some mammals

Age of animals was 3-6 years. Average values, \pm S. D., for the indicated number of animals are given. Sialic acid denotes *N*-acetyl-neuraminic (*N*-acet.) and *N*-glycolylneuraminic (*N*-glyc.) acids; hex./sial., the ratio of hexoses to sialic acid.

Family	Species	No. of ani- mals	Ratio: N-acet./ N-glyc.	In total protein				Ratio: hex./sial.	In seromuroid				Ratio: hex./sial.
				Hexoses		Sialic acid			Hexoses		Sialic acid		
				mg./100 ml. serum	g./100 g. protein	mg./100 ml. serum	g./100 g. protein		mg./100 ml. serum	g./100 g. protein	mg./100 ml. serum	g./100 g. protein	
Bovidae	Buffalo, <i>Bubalus bubalus</i>	1	1.1	118	1.7	57	0.8	2.1	7.0	12.0	6.5	10.9	1.1
	European bison, <i>bison bonasus</i>	3	1.0	109 ±12.0	1.8 ±0	60 ±4.7	1.0 ±0	1.8 ±0.05	7.8 ±0.37	12.0 ±0.30	6.6 ±0.28	10.1 ±0.82	1.2 ±0.10
Felidae	Lion, <i>Panthera leo</i>	4	6.2	118 ±10.7	1.9 ±0.12	65 ±3.2	1.0 ±0.05	1.8 ±0.20	7.3 ±1.09	12.2 ±0.56	5.3 ±0.33	8.9 ±0.80	1.4 ±0.15
	Tiger, <i>Panthera tigris</i>	1	7.3	128	2.4	84	1.6	1.5	11.4	12.2	8.2	8.8	1.4
Cervidae	Fallow deer, <i>Dama dama</i>	7	3.8	71 ±1.4	1.5 ±0.06	45 ±1.7	1.0 ±0.06	1.6 ±0.08	5.7 ±0.48	10.3 ±0.59	3.3 ±0.32	6.0 ±0.83	1.7 ±0.28
	Red deer, <i>Cervus elaphus</i>	2	—	83	1.5	58*	1.0*	1.5	11.1	9.7	6.8*	5.9*	1.6
Ursidae	Brown bear, <i>Ursus arctos</i>	6	—	98 ±9.4	1.5 ±0.06	62* ±1.6	0.9* ±0	1.6 ±0.17	9.9 ±0.59	15.1 ±0.7	5.1* ±0.11	7.7* ±0.74	2.0 ±0.13
Leporidae	European brown hare, <i>Lepus europaeus</i>	14	5.7	77 ±2.8	1.8 ±0.07	37 ±4.6	0.9 ±0.16	2.1 ±0.10	12.5 ±2.21	12.5 ±0.09	6.2 ±0.60	6.2 ±1.16	2.0 ±0.11

* Calculated as *N*-acetylneuraminic acid.

similar, especially the ratio between the two neuraminic acid derivatives in total serum protein, the content of hexoses and sialic acids in seromucoids and the ratio of hexoses to sialic acid.

DISCUSSION

The obtained results do not fully confirm our previous suggestion [3] that the electrophoretical mobility and distribution of glycoprotein fractions in mammals are species-dependent. Although in some families, i.e. Cervidae and Leporidae, large differences in electrophoretical patterns between animal species were found even when the animals were morphologically similar, e.g. rabbit and hare, within other families, i.e. Bovidae and Felidae, the electrophoretical patterns exhibited marked similarities.

In the present estimations, similarly as in the previous ones [3], marked differences were found in the content of carbohydrates bound with total protein per 100 ml. of serum and in their content in protein, between different species of animals irrespective of the family they belong to. Therefore it seems that these properties are species-dependent. In a similar study on four species of domesticated animals Montreuil *et al.* [6] also found marked species-dependent differences in the content of carbohydrates in "total glycoproteins" of blood serum.

On the other hand, in seromucoids the content of hexoses and sialic acid as well as the ratio of hexoses to sialic acid were similar to a marked degree in animals belonging to the same family.

Marked differences in the ratio of *N*-acetylneuraminic acid to *N*-glycolylneuraminic acid found in serum proteins of non-related animal species were also reported by Martensson *et al.* [5] for four domesticated species; this ratio was especially low in cattle. Similarly low ratios were now obtained for species belonging to the same family, i.e. european bison and buffalo.

In hares the level in serum of hexoses bound with total protein was much lower than that found in the previous work [3]. The content of hexoses in protein was, however, almost the same so the difference seems to be due to a decrease in the protein level in the serum. The experiments reported in the present work were performed during the extremely cold winter of 1962/1963 when wild animals suffered particular hardships, and only the strongest and most resistant individuals were able to survive. It seems that the observed difference might have been due to these circumstances. It is worth noting that the standard deviation in the present estimations was very low as contrasted with high S. D. values obtained for hare sera examined previously [3].

The content of sialic acid in seromucoids was calculated on the basis of sialic acid content in total serum protein, and in bear and red deer

as *N*-acetylneuraminic acid. Nevertheless it is possible that in sero-mucoids the neuraminic acid derivatives are present in a different ratio and this might have affected the accuracy of the presented results.

SUMMARY

Serum glycoproteins of various species of non-domesticated mammals were studied. Electrophoretic patterns of glycoprotein fractions exhibited in some species family-dependent similarities. Species-dependent differences were found in the content of hexoses and sialic acid in total serum proteins and in the ratio of hexoses to sialic acid.

REFERENCES

- [1] Dżułyńska J. & Piekarska Z. - *Bull. Acad. Pol. Sci. Ser. Biol.* **4**, 419, 1956.
- [2] Dżułyńska J. & Piekarska Z. - *Bull. Acad. Pol. Sci. Ser. Biol.* **6**, 133, 1958.
- [3] Dżułyńska J., Lutowicz J. & Kędzierska B. - *Acta Biochim. Polon.* **9**, 391, 1962.
- [4] Klenk E. & Uhlenbruck G. - *Z. Phys. Chem.* **307**, 266, 1957.
- [5] Martensson E., Raal A. & Svennerholm L. - *Biochim. Biophys. Acta* **36**, 124, 1958.
- [6] Montreuil J., Seynave R., de Souza B. C. & Hivet D. - *C. R. Acad. Sci.* **254**, 1530, 1962.
- [7] Svennerholm L. - *Acta Soc. Med. Ups.* **61**, 75, 1956.
- [8] Wael de J. & Punt K. - *Clin. Chim. Acta* **2**, 403, 1957.
- [9] Weichselbaum T. E. - *Am. J. Clin. Path.*, techn. section, **10**, 40, 1946.
- [10] Wieland T. & Fischer E. - *Naturwissenschaften* **35**, 29, 1948.
- [11] Winzler R. J. in *The Plasma Proteins* (F. W. Putnam, ed.), vol. 1, p. 315. Academic Press, New York-London, 1960.

GLIKOPROTEINY SUROWICY NIEKTÓRYCH GATUNKÓW SSAKÓW NIEUDOMOWIONYCH

Streszczenie

Przeprowadzono badania nad glikoproteinami surowicy różnych gatunków ssaków nieudomowionych. Wśród niektórych gatunków stwierdzono występowanie podobieństw rodzinnych w zachowaniu się elektroforetycznych frakcji glikoproteinowych. Badania chemiczne heksoz i kwasów sjalinowych wskazują na istnienie różnic gatunkowych w zawartości badanych węglowodanów w białkach całkowitych surowicy i wartości wzajemnego ich stosunku.

Received 4 November 1963.

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SERUM GLYCOPROTEINS IN SOME SPECIES OF BIRDS*Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa*

So far the comparative studies on animal serum glycoproteins were restricted almost exclusively to mammals [4, 5, 3, 2, 1] and in the available literature we could find no data concerning the birds. Therefore we have examined the sera of some birds and the presented results seem to indicate species-dependent differences in serum glycoproteins. Some properties, however, were found to be common to all birds studied but different from properties observed in mammals.

MATERIALS AND METHODS

Blood samples for chemical and electrophoretical estimations were taken by puncture of the jugular vein from the domestic duck, goose and fowl, and from the turkey, pigeon and guinea fowl. Blood samples for isolation of sialic acid were taken in a slaughterhouse.

Paper electrophoresis. This was performed in the same way as in the previous work [1] except that 15-20 μ l. samples of serum were applied for protein staining; this was found necessary because the content of serum proteins is lower in birds than in mammals.

Chemical analysis and chromatography. The methods used were the same as in the previous work [1].

RESULTS

The results of electrophoretical separation of bird serum glycoproteins are presented in Table 1. Differences between the species were found not only in the mobility of glycoprotein fractions but also in their quantitative distribution, and even in the number of fractions.

The greatest amounts of substances staining with Schiff's reagent were present in α -globulins; however, also other fractions were sometimes rich in glycoproteins, e.g. albumins in the pigeon and γ -globulins in the pigeon and turkey. Some glycoprotein fractions present in human

Table 1

Electrophoretic mobility and distribution of serum glycoprotein fractions in six species of birds

Average value of 6 determinations from 3 animals are given; in parentheses the limit values. Mobility is expressed as $\mu = \text{cm}^2/\text{sec.}/\text{volt} \times 10^5$. Normal human serum from one donor was used as reference.

Species	Albumin		α_1		α_2		β		γ	
	Mobility (μ)	Distribution (%)	Mobility (μ)	Distribution (%)	Mobility (μ)	Distribution (%)	Mobility (μ)	Distribution (%)	Mobility (μ)	Distribution (%)
Dom. duck, <i>Anas platyrhynchos</i>	5.4 (5.3 - 5.6)	18 (15 - 22)	4.0* (3.8 - 4.2)	52 (45 - 60)	0	0	2.7 (2.5 - 2.8)	19 (18 - 21)	1.9 (1.8 - 2.1)	11 (8 - 12)
Dom. goose, <i>Anser anser</i>	5.6 (5.4 - 5.7)	13 (13 - 13)	4.5 (4.4 - 4.5)	24 (24 - 25)	3.7 (3.7 - 3.8)	30 (29 - 32)	2.8 (2.6 - 2.9)	21 (19 - 22)	2.0 (1.8 - 2.1)	12 (11 - 12)
Dom. fowl, <i>Gallus gallus</i>	5.8 (5.7 - 5.9)	7 (6 - 8)	4.7 (4.5 - 5.0)	34 (31 - 35)	3.6 (3.5 - 3.8)	21 (17 - 25)	2.3** (2.2 - 2.3)	38 (35 - 42)	0	0
Turkey, <i>Melleagris gallopavo</i>	5.3 (5.3 - 5.3)	21 (20 - 21)	3.9*** (3.8 - 3.9)	36 (32 - 39)	0	0	2.7 (2.6 - 2.8)	14 (13 - 16)	2.0 (1.9 - 2.2)	29 (27 - 32)
Pigeon, <i>Columba livia</i>	5.1 (4.9 - 5.3)	33 (30 - 36)	3.8 (3.8 - 3.9)	21 (18 - 27)	3.0 (2.9 - 3.1)	8 (6 - 9)	2.4 (2.4 - 2.5)	13 (11 - 17)	1.8 (1.8 - 1.8)	25 (21 - 32)
Guinea fowl, <i>Numida melleagris</i>	5.6 (5.5 - 5.6)	11 (9 - 15)	4.3 (4.2 - 4.5)	37 (37 - 39)	3.5 (3.5 - 3.6)	17 (17 - 18)	2.8 (2.7 - 2.9)	20 (19 - 21)	1.8 (1.7 - 1.8)	15 (12 - 17)
Man	5.9	6	4.6	17	3.7	32	2.9	27	1.5	18

* α_1 -fraction.** Glycoprotein fraction with mobility intermediate between that for β - and γ -protein fractions.*** Glycoprotein fraction with mobility intermediate between that for α_1 - and α_2 -protein fractions.

Protein-bound hexoses and sialic acid in serum of six species of birds

Age of animals was 6-9 months. Average values, \pm S. D., for the indicated number of animals are given; in parentheses, the limits of the confidence interval. Sialic acid denotes *N*-acetylneuraminic and *N*-glycolylneuraminic acids; hex./sial., the ratio of hexoses to sialic acid.

Species	No. of animals	Hexoses		Sialic acid		Ratio: hex./sial.
		mg./100 ml. serum	g./100 g. protein	mg./100 ml. serum	g./100 g. protein	
In total protein						
Dom. duck, <i>Anas platyrhynchos</i>	11	58.6 ± 4.9 (54.9; 62.3)	1.9 ± 0.07 (1.84; 1.94)	28.2 ± 1.5 (27.0; 29.4)	0.90 ± 0.05 (0.87; 0.93)	2.08 ± 0.15 (1.97; 2.19)
Dom. goose, <i>Anser anser</i>	27	82.3 ± 2.8 (81.1; 83.4)	2.08 ± 0.08 (2.04; 2.11)	41.6 ± 1.9 (40.8; 42.4)	1.07 ± 0.05 (1.05; 1.09)	1.99 ± 0.05 (1.97; 2.01)
Dom. fowl, <i>Gallus gallus</i>	33	105.3 ± 8.9 (102.1; 108.4)	2.75 ± 0.06 (2.73; 2.77)	47.4 ± 3.1 (46.3; 48.5)	1.23 ± 0.04 (1.21; 1.24)	2.24 ± 0.09 (2.20; 2.27)
Turkey, <i>Melleagris gallopavo</i>	12	108.7 ± 8.6 (103.0; 114.5)	2.37 ± 0.11 (2.30; 2.45)	46.7 ± 3.0 (44.7; 48.7)	1.03 ± 0.06 (0.99; 1.07)	2.37 ± 0.15 (2.27; 2.48)
Pigeon, <i>Columba livia</i>	12	71.8 ± 0.7 (71.4; 72.3)	2.54 ± 0.05 (2.51; 2.58)	32.6 ± 1.0 (32.0; 33.2)	1.15 ± 0.05 (1.12; 1.18)	2.21 ± 0.08 (2.16; 2.26)
Guinea fowl, <i>Numida melleagris</i>	4	80.5 ± 2.8 (75.4; 85.6)	2.92 ± 0.08 (2.77; 3.08)	38.3 ± 2.1 (34.5; 42.0)	1.40 ± 0.07 (1.27; 1.53)	2.10 ± 0.07 (1.97; 2.23)
In serum						
Dom. duck, <i>Anas platyrhynchos</i>	11	6.45 ± 0.82 (5.83; 7.07)	7.22 ± 0.34 (6.97; 7.47)	3.08 ± 0.25 (2.89; 3.27)	3.45 ± 0.35 (3.18; 3.72)	2.08 ± 0.13 (1.98; 2.18)
Dom. goose, <i>Anser anser</i>	27	8.37 ± 0.69 (8.10; 8.65)	10.92 ± 0.74 (10.62; 11.22)	4.23 ± 0.38 (4.07; 4.38)	5.53 ± 0.48 (5.34; 5.72)	1.99 ± 0.18 (1.91; 2.06)
Dom. fowl, <i>Gallus gallus</i>	33	14.21 ± 0.65 (13.84; 14.58)	14.51 ± 0.66 (14.13; 14.89)	7.11 ± 0.29 (6.94; 7.27)	7.29 ± 0.36 (7.08; 7.49)	2.00 ± 0.04 (1.98; 2.02)
Turkey, <i>Melleagris gallopavo</i>	12	14.13 ± 1.11 (13.34; 14.91)	15.70 ± 0.71 (15.20; 16.20)	6.61 ± 0.27 (6.42; 6.80)	7.38 ± 0.25 (7.20; 7.56)	2.02 ± 0.08 (1.96; 2.08)
Pigeon, <i>Columba livia</i>	—	—	—	—	—	—
Guinea fowl, <i>Numida melleagris</i>	4	14.86 ± 0.66 (12.85; 16.87)	11.43 ± 0.86 (8.83; 14.03)	7.16 ± 0.05 (7.02; 7.30)	5.70 ± 0.16 (5.20; 6.20)	2.03 ± 0.09 (1.74; 2.32)

serum were absent from the sera of some species of birds. For instance in the duck only 4 fractions of proteins and of substances staining with Schiff's reagent were found (Fig. 1). In other birds, although protein

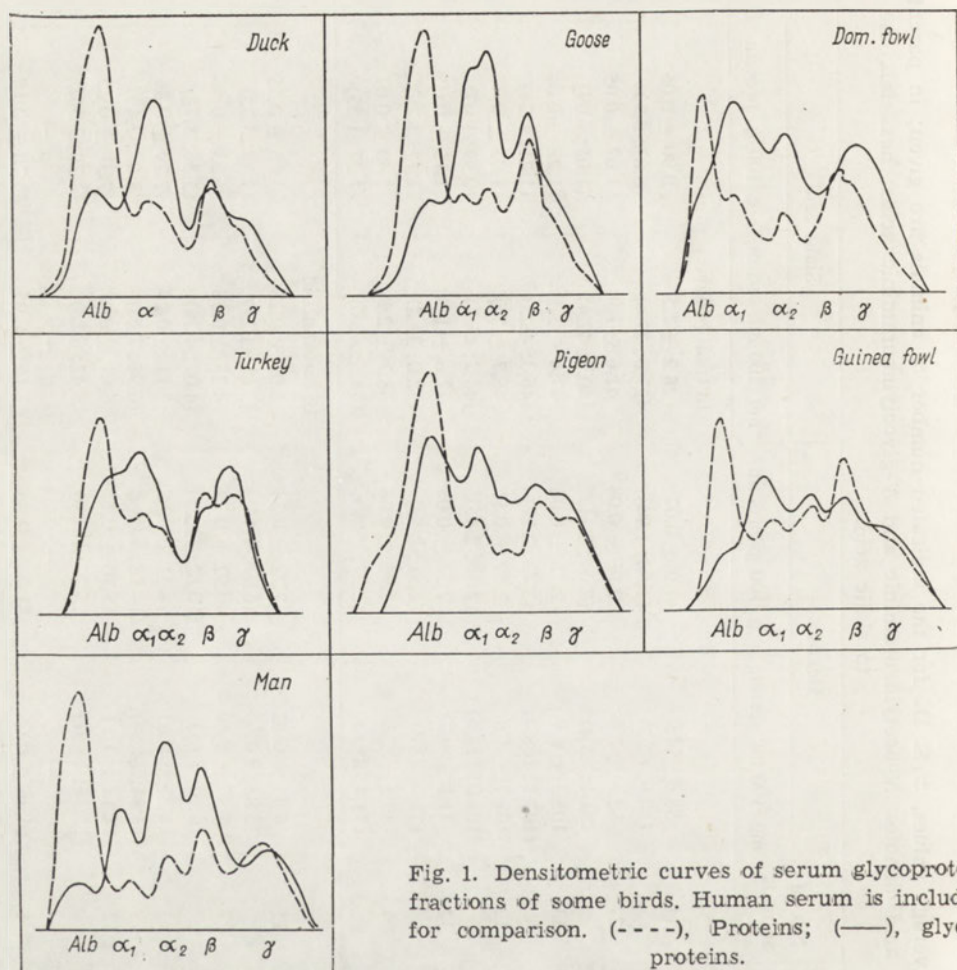


Fig. 1. Densitometric curves of serum glycoprotein fractions of some birds. Human serum is included for comparison. (---), Proteins; (—), glycoproteins.

separated into 5 fractions, only 4 glycoproteins were obtained. In the domestic fowl at the place of β - and γ -globulin fractions situated in close proximity, only one band staining with Schiff's reagent was consistently obtained. Similarly in the turkey, at the place of α_1 - and α_2 -globulins only one glycoprotein fraction was found.

Although only six species of birds were examined in the present work, the obtained results seem to indicate that the mobility and quantitative distribution of serum glycoprotein fractions in birds are species-dependent.

Further data on bird serum glycoproteins were obtained by chemical assays of hexoses and sialic acid bound with total protein and sero-

mucoids. In some species, i.e. domestic fowl, duck, goose and turkey, sialic acid was also isolated from serum protein and subjected to paper chromatography; in each serum only one spot was found with the R_F value corresponding to that of the simultaneously run standard of *N*-acetylneuraminic acid. The more sensitive chemical method [2] showed also the presence of *N*-glycolylneuraminic acid, but only in traces.

Marked differences in the content of hexoses and sialic acid bound with total serum protein were found in the examined species of birds (Table 2). In the domestic fowl and turkey the content of carbohydrates was relatively high; hexoses on the average 105 and 109 mg./100 ml. of serum, resp., and sialic acid 47 mg./100 ml.; in the duck, on the other hand, only 59 mg. of hexoses and 28 mg. of sialic acid per 100 ml. were found. The differences in the content of carbohydrates per 100 g. of protein were smaller but still distinct.

Marked species-dependent differences were also found in the content of hexoses and sialic acid in seromucoids calculated per 100 ml. of serum and per 100 g. of protein.

While the contents of the tested protein-bound compounds in the sera vary with the species of birds, the ratio of hexoses to sialic acid is almost the same in seromucoids and differs but slightly in total proteins.

DISCUSSION

Glycoprotein fractions of bird sera were named by us tentatively after human serum fractions, although the mobilities of bird serum fractions differed sometimes largely from the analogously called human ones. In the pigeon the fraction succeeding albumins, which we have called the α_1 -glycoprotein fraction, had almost the same mobility as the α_2 -glycoprotein of human serum, while α_2 - in birds moved similarly as the β - in human serum.

Although marked differences between the species were observed in the mobility and distribution of glycoprotein fractions, some properties seem to be common to all the species examined. In birds the γ -glycoprotein fraction moved much faster than the corresponding human one, and the content of carbohydrates in total serum protein was markedly higher than in mammals [2]. In all the birds tested the ratio of hexoses to sialic acid in seromucoids was almost identical; this might suggest that the stability of the composition of the polysaccharide component in these glycoproteins is a characteristic feature of bird sera.

SUMMARY

Serum glycoproteins of 6 species of birds were studied. Electrophoretic patterns and the content of carbohydrates in total serum protein

and in seromucoids exhibited species-dependent differences. On the other hand the ratios of hexoses to sialic acid were very similar in all species and the content of carbohydrates in sera was much higher than that found in most mammals.

REFERENCES

- [1] Dżułyńska J., Krajewska K. & Gill J. - *Acta Biochim. Polon.* **11**, 121, 1964.
- [2] Dżułyńska J., Lutowicz J. & Kędzierska B. - *Acta Biochim. Polon.* **9**, 391, 1962.
- [3] Montreuil J., Seynave R., de Souza B. C. & Hivet D. - *C. R. Acad. Sci.* **254**, 1530, 1962.
- [4] Sohar E., Bossak E. T. & Adlersberg D. - *Proc. Soc. Exp. Biol. Med.* **90**, 305, 1955.
- [5] Weimer A. E. & Winzler R. J. - *Proc. Soc. Exp. Biol. Med.* **90**, 459, 1955.

GLIKOPROTEINY SUROWICY NIEKTÓRYCH GATUNKÓW PTAKÓW

Streszczenie

Przeprowadzono badania nad glikoproteinami surowicy niektórych gatunków ptaków. Otrzymane wyniki wskazują na występowanie różnic gatunkowych w zachowaniu się elektroforetycznych frakcji glikoproteinowych i zawartości węglowodanów w białkach całkowitych surowicy i seromukoidach; ponadto u wszystkich badanych gatunków stwierdzono istnienie pewnych wspólnych własności glikoprotein rzadko spotykanych w surowicach ssaków.

Received 12 November 1963.

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UTILIZATION OF [1-¹⁴C]GLYCINE IN PURINE BIOSYNTHESIS IN *HELIX POMATIA*

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The pathway of uric acid synthesis in snails is not yet fully elucidated. The results of Bricteux-Grégoire & Florkin [1, 2] who injected ¹⁴C-labelled urea into the hepatopancreas of *Helix pomatia* plead against urea being a precursor of uric acid. Only 1% of the applied radioactivity was found in uric acid, and this may be accounted for by CO₂ derived from urea degradation. Sixty to seventy percent of the incorporated activity was found in carbon 6 known to arise from CO₂.

In our previous communications [7, 8] we have demonstrated that the snail *Helix pomatia* is uricotelic throughout the whole year. Over 90% of its nitrogen wastes consists of purines: uric acid, xanthine, and usually also guanine. In the present work, the incorporation of glycine labelled with ¹⁴C in the carboxyl group into the purine ring in *Helix pomatia* was studied. Into the hepatopancreas of two adult hibernating snails 2-4 µc glycine (specific activity 8.3 mc/10 m-moles, Amersham Radiochemical Centre, England) was injected. After 4 days the nephridium was isolated and the nephridial contents rinsed out with 20 ml. of water. The obtained suspension was centrifuged for 15 min. at 6000 r.p.m. The purines sedimented at the bottom of the tube, and over them settled a layer of nephridial fragments. The supernatant fluid was decanted together with the nephridial fragments, boiled, and the sedimented protein centrifuged off. The fluid was combined with the sedimented purines which were dissolved by heating with lithium carbonate (0.5 ml. of sat. soln.) water being added to final volume of 100 ml. From this solution purines were separated by chromatography on Whatman no. 3 paper in 60% *n*-propanol. The spots of purines were located in UV light, a 280-300 mµ filter being used. The corresponding areas were cut out from the chromatogram and eluted with hot water. In the eluates, uric acid was assayed colorimetrically [5] and xanthine and guanine spectrophotometrically [10]; the radioactivity of each of the purine compounds

was assayed on samples evaporated in tin planchets, with a G-M counter (mica window, 1.2 mg./cm.²).

Two snails were used for this experiment. The first one was injected with radioactive glycine (4 μ c); uric acid and xanthine found in the nephridium contained 11.4% of the radioactivity dosis. The second snail was injected with 2 μ c; three purines present in the nephridium contained 8.8% of the radioactivity dosis (Table 1). These results indicate that glycine participates in the synthesis of the purine ring in *Helix pomatia*.

Table 1

Incorporation of radioactivity into the purine ring in Helix pomatia

Glycine labelled with ¹⁴C in the carboxyl group was injected into the hepatopancreas of hibernating snail, 4 μ c (480 000 counts/min.) in expt. I, 2 μ c (240 000 counts/min.) in expt. II. After 4 days the contents of nephridium were examined.

Purines	Counts/min.	Counts/min./ μ mole	% of the dosis
Expt. I			
Uric acid	28 420	179	5.9
Xanthine	26 550	155	5.5
Sum	54 970		11.4
Expt. II			
Uric acid	10 940	133	4.6
Xanthine	7 160	98	3.0
Guanine	2 930	65	1.2
Sum	21 030		8.8

To establish the position into which the ¹⁴C of glycine is incorporated, 1 μ c of [1-¹⁴C]glycine was injected into the hepatopancreas of snail during the feeding period. For the next two weeks the nephridial excreta were collected. They were dissolved in hot water and from the solution uric acid was crystallized several times; this procedure served to remove xanthine which is 50 times more soluble in water than uric acid. In these excreta no guanine was found. The isolated uric acid was assayed for radioactivity and then degraded by heating 6 mg. with 0.5 ml. of concentrated hydrochloric acid in a sealed glass tube at 160° for 18 hr. [9]. Under these conditions the purine ring is broken in such a way that glycine is formed again from carbon 4 and 5, and nitrogen 7. After hydrolysis the solution was evaporated almost to dryness, then distilled water was added. The evaporation was repeated several times to remove hydrochloric acid. Then the solution was adjusted to pH 8 with dilute sodium hydroxide and boiled to remove ammonia. The solution was applied as a band on Whatman no. 4 paper and chromatographed in

water [4]. Glycine was located with ninhydrin on a narrow strip of the chromatogram and the corresponding areas were cut out and eluted. In the eluates the radioactivity was assayed as described above and the amount of glycine was determined by the method of Jacobs [6]. The specific radioactivity of glycine was compared to that of uric acid isolated from the nephridial excreta (Table 2). In two parallel experiments, 62 and 77% radioactivity, respectively, was found in the position corresponding to carbon 4 or 5. About 30% of the label remained in other positions; this can be accounted for by metabolic degradation of glycine into formate and bicarbonate which is expected to occur in longer-term experiments.

Table 2

Radioactivity of carbon 4 and 5 of uric acid isolated from nephridial excreta of Helix pomatia

Into the hepatopancreas of a snail during the feeding period 1 μ c [1- 14 C]glycine (8.3 mc/10 m-moles) was injected. From nephridial excreta collected during two weeks uric acid was isolated and decomposed to glycine.

Expt. no.	Counts/min./ m-mole uric acid	Counts/min./ m-mole glycine	% of radioactivity of uric acid at positions 4 and 5
1	38 500	29 800	77.4
2	25 300	15 700	62.0

The presented experiments indicate that in *Helix pomatia* glycine is incorporated into the same positions as those established by Buchanan for the biosynthesis of the purine ring in pigeons and confirmed in other animals [3]. Therefore, in agreement with the results of Bricteux-Grégoire & Florkin who after administration of labelled urea found the label in carbon 6, it might be supposed that the pathway of purine biosynthesis is the same in *Helix pomatia* as in other organisms.

SUMMARY

[1- 14 C]Glycine was injected into the hepatopancreas of the snail *Helix pomatia*. After 4 days about 10% of the radioactivity dosis was found in the excreted purine compounds. About 70% of the total radioactivity of uric acid was found in carbon 4 or 5.

REFERENCES

- [1] Bricteux-Grégoire S. & Florkin M. - *Arch. Intern. Physiol. Biochim.* **70**, 144, 1962.
- [2] Bricteux-Grégoire S. & Florkin M. - *Arch. Intern. Physiol. Biochim.* **70**, 496, 1962.

- [3] Buchanan J. M. & Hartman S. C. - *Advanc. Enzymol.* **21**, 199, 1959.
- [4] Burma D. P. & Banerjee B. - *Science and Culture* (India) **15**, 363, 1950.
- [5] Caraway W. T. - *Am. J. Clin. Path.* **25**, 7, 840, 1955.
- [6] Jacobs S., XV Congresso Internacional de Quimica Pura e Aplicada *Actas de Congresso* vol. 3, VIII - 20, Lisboa 1956.
- [7] Jeżewska M. M., Gorzkowski B. & Heller J. - *Acta Biochim. Polon.* **10**, 55, 1963.
- [8] Jeżewska M. M., Gorzkowski B. & Heller J. - *Acta Biochim. Polon.* **10**, 309, 1963.
- [9] Korn E. D., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. IV, p. 615. Academic Press, New York 1957.
- [10] Plesner P. & Kalckar H. M., in *Methods of Biochemical Analysis* (ed. D. Glick) vol. III, p. 97. Interscience Publishers, New York 1956.

WYKORZYSTANIE [1-¹⁴C]GLICYNINY W BIOSYNTEZIE PIERSCIENIA PURYNOWEGO U *HELIX POMATIA*

Streszczenie

Wstrzykiwano [1-¹⁴C]glicynę w hepatopancreas ślimakom *Helix pomatia*. Stwierdzono, że po czterech dniach ok. 10% podanej radioaktywności znajduje się w wydalanych purynach. Węgiel z pozycji 4 lub 5 zawierał ok. 70% całkowitej radioaktywności kwasu moczowego.

Received 21 November 1963.

HELENA ZAŁĘSKA and R. PAKUŁA

**BIOLOGICAL EFFECTS OF RENATURATION OF MIXTURES
OF GENETICALLY DIFFERENT DNA'S OF*****BACILLUS SUBTILIS***

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Exposure of solutions of native DNA to elevated temperatures above the melting point results in the rupture of the inter-strand hydrogen bonds followed by separation of the strands and in loss of transforming activity [5, 6]. Slow cooling reunites the strands so as to restore a large part of the transforming activity. This process is known as renaturation [2, 7]. The degree of renaturation is dependent on concentration of DNA in solution, on ionic strength of the solute and on temperature. A high degree of renaturation can be obtained if the denatured DNA is maintained for 2 hr. at a temperature by about 25° lower than the temperature of the melting point.

By heating and slow cooling (annealing) of mixtures of DNA from two mutants, carrying two different markers, of the same species, or from various but related species, hybrid DNA molecules can be produced. In the hybrid DNA, each strand stems from a different source. Complementarity is a necessary condition for combining strands from various sources. Some deviation from complementarity, however, seems not to interfere with formation of hybrid molecules. Strands coming from DNA's extracted from mutants of the same species can obviously be regarded as complementary ones. Schildkraut, Marmur & Doty [10] have shown that renaturation of mixture of two DNA's results in production of hybrid molecules in a proportion determined by random collisions of complementary strands. When a ¹⁵N-labelled DNA was mixed 1:1 with non-labelled light DNA, the renatured nucleic acid was composed of heavy, hybrid and light DNA in a proportion 1:2:1 as was shown by density gradient centrifugation in caesium chloride.

The biological activity of hybrid DNA's was first investigated by Herriott [3] who renatured two DNA's extracted from two mutants of *Haemophilus influenzae*, one resistant to streptomycin and the other

resistant to cathomycin. In *Haemophilus influenzae* these markers were found to be linked. The exposure of the sensitive, recipient population to the renatured mixture of DNA resulted in production of transformants resistant to either of the two antibiotics and to both of them. Although the number of double transformations was only about 1% of the single ones, it was much above the number produced by a mixture of the same DNA's renatured separately and above the number expected from a random distribution of genetic units carrying the respective markers. Thus heat denaturation of a mixture of genetically different DNA's from different mutants of *Haemophilus influenzae* led to formation of heterozygotes carrying both genetic markers.

Different results were obtained by Marmur, Rownd & Schildkraut [8] with renatured mixtures of transforming, pneumococcal DNA. The DNA's carried the linked, ery₂ and ery₃, erythromycin-resistant markers. No double transformants were found with the hybrid DNA.

The aim of this study was to test the transforming activity of hybrid DNA of *Bacillus subtilis* and to see whether, like in *Haemophilus influenzae*, double transformations can be produced with this DNA. *Bacillus subtilis* seemed to be a proper microorganism for this kind of investigation because of the close linkage of two markers namely those of indole and tyrosine synthesis.

MATERIALS AND METHODS

Strains. The recipient and donor strains of *Bacillus subtilis* were received from the Microbial Genetic Research Unit in Hammersmith Hospital in London. As recipient we used the nutritionally deficient mutant with a growth requirement for tryptophan or indole and tyrosine (ind⁻,tyr⁻). The mutant requiring indole was designated ind⁻,tyr⁺ and that requiring tyrosine ind⁺,tyr⁻. The prototrophic wild type strain was designated ind⁺,tyr⁺. The strains were preserved at 4° on nutrient agar.

Preparation of transforming DNA. DNA for transformation was prepared according to the method of Marmur [4]. Donor strains were cultivated in the fluid medium of Anagnostopoulos & Spizizen [1, 11]. This medium was supplemented with either indole or tyrosine when the respective biochemical deficient mutants were grown for DNA extraction. Five-liter bottles were filled with 1 liter of medium, the medium was inoculated and the culture allowed to grow under continual shaking for 18 hr. at 37°. The culture was then centrifuged, the bacteria suspended in 0.1 M-sodium versenate - 0.15 M-NaCl (pH 8.0) and exposed to the action of lysozyme for 60 min. Dodecyl-sulphate was next added, protein removed by repeated shaking with chloroform and DNA precipitated by addition of 2 volumes of ethanol. The nucleic acid was dissolved in saline so as to obtain 400 - 600 µg./ml. of DNA. As recommended by Spizizen [11], the

DNA preparations were not treated with RNase. The DNA content in the preparations of the transforming principle was determined according to the method of Stumpf [12].

Heat denaturation and renaturation of DNA. For denaturation, stocks of DNA were diluted with 0.3 M-NaCl - 0.03 M-sodium citrate to a concentration of 25 $\mu\text{g./ml.}$; 1.5 ml. samples of these solutions were poured into 15 \times 150 mm. Pyrex tubes and covered with 0.5 ml. of paraffin oil to avoid evaporation. The tubes were then maintained for 10 min. in a boiling water bath and either cooled rapidly in an ice bath or, for renaturation, kept for 2 hr. at 65° and then slowly cooled to ambient temperature.

Transformation procedure. The media I and II of Spizizen [1, 11] were used for transformation; 12 ml. portions of medium I were poured into 100 ml. Erlenmeyer flasks and inoculated with 10% of an 18 hr. old culture of the recipient strain. The bacteria were grown under continuous shaking for 4 hr. and then samples of this culture were diluted 4 times with medium II. After 1.5 hr. of cultivation, DNA was added. Non absorbed DNA after 30 min. was destroyed by addition of DNase activated with Mg^{2+} . Properly diluted 0.5 ml. aliquots of the culture were immediately spread on Petri plates containing the selective agar medium. It was the minimal medium of Spizizen with 1% glucose for scoring $\text{ind}^+, \text{tyr}^+$ transformants. This medium was supplemented with either indole or tyrosine when $\text{ind}^-, \text{tyr}^+$ and $\text{ind}^+, \text{tyr}^-$ transformants were scored. For control Petri plates were seeded with culture samples of the recipient strain not exposed to the action of DNA or treated with DNA previously destroyed by DNase.

RESULTS

Figure 1 illustrates the biological effects of denaturation and renaturation of transforming DNA extracted from an $\text{ind}^+, \text{tyr}^-$ mutant of *B. subtilis*. The recipient strain was $\text{ind}^-, \text{tyr}^-$. Transformations were carried out with various concentrations of DNA. As can be seen in Fig. 1, up to about 3×10^{-2} $\mu\text{g./ml.}$ of DNA the number of transformants is proportional to the concentration of the transforming principle. Heating of DNA at 100°, under conditions described above, followed by rapid cooling, resulted in a loss of about 99% of initial transforming activity. Slow cooling of denatured DNA restored, in various experiments 20 - 60% of initial activity. Similar results were obtained when the $\text{ind}^-, \text{tyr}^-$ recipient cells were transformed by proper DNA to $\text{ind}^-, \text{tyr}^+$ or to prototrophy ($\text{ind}^+, \text{tyr}^+$) by wild type DNA.

Figure 2 shows the degree of linkage between the two markers under test. With $\text{ind}^-, \text{tyr}^-$ recipient cells and $\text{ind}^+, \text{tyr}^+$ transforming DNA, the number of double transformants was about 40 - 45% of the number

of singles $\text{ind}^+, \text{tyr}^-$ and $\text{ind}^-, \text{tyr}^+$. In most cases, no large differences between the number of both kinds of single transformations were observed.

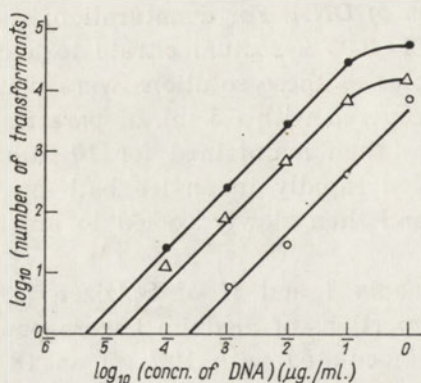


Fig. 1

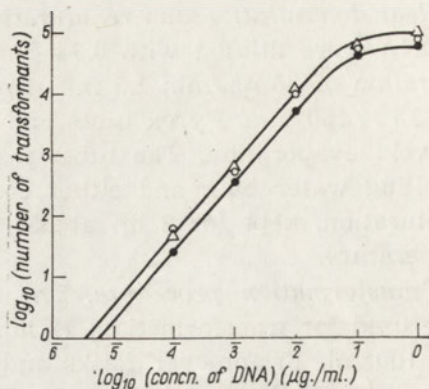


Fig. 2

Fig. 1. The effect of denaturation and renaturation of DNA on efficiency of transformation. Recipient: *B. subtilis* $\text{ind}^-, \text{tyr}^-$. Donor: *B. subtilis* $\text{ind}^+, \text{tyr}^-$. (●), Native DNA; (Δ), renatured DNA; (○), denatured DNA.

Fig. 2. Efficiency of double and single transformants. Recipient: *B. subtilis* $\text{ind}^-, \text{tyr}^-$. Donor: *B. subtilis* $\text{ind}^+, \text{tyr}^+$. (○), $\text{ind}^-, \text{tyr}^+$ transformants; (Δ), $\text{ind}^+, \text{tyr}^-$ transformants; (●), $\text{ind}^+, \text{tyr}^+$ transformants.

In the following experiment illustrated in Table 1 $\text{ind}^+, \text{tyr}^-$ DNA and $\text{ind}^-, \text{tyr}^+$ DNA were either separately denatured and renatured in a proportion 1:3 or mixed together in the same proportion before denaturation. Aliquots of transforming DNA treated in this way as also aliquots of the two DNA's mixed 1:3 not heated at all were used for transformation of $\text{ind}^-, \text{tyr}^-$ recipient cells. Scoring was made for single transformants $\text{ind}^+, \text{tyr}^-$ and $\text{ind}^-, \text{tyr}^+$ as also for doubles $\text{ind}^+, \text{tyr}^+$. As can be seen in Table 1, the $\text{ind}^+, \text{tyr}^-$ and $\text{ind}^-, \text{tyr}^+$ DNA's mixed before renaturation do not induce double transformants like the same DNA's mixed after renaturation. The separation of the complementary strands in the DNA's was probably satisfactory because the residual transforming activity after denaturation was less than 1%. As concerns single transformations, there were also no significant differences in activity of DNA preparations heated and annealed before mixing or renatured together after mixing.

The activity of each marker of the non-heated mixture (0.01 $\mu\text{g./ml.}$ of $\text{ind}^+, \text{tyr}^-$ DNA and 0.03 $\mu\text{g./ml.}$ of $\text{ind}^-, \text{tyr}^+$ DNA) was by 35–40% lower than the activity of each of these DNA's used separately. We have no explanation for the drop of activity when the DNA's are used in mixture. The possibility of mutual inhibition of each marker by the other DNA cannot be excluded although low concentrations of DNA were used for transformation.

Table 1

The effect of renaturation in mixture of genetically different DNA's

Recipient: *B. subtilis* ind⁻, tyr⁻.

DNA of donors used in transformation	Number of transformants		
	ind ⁺	tyr ⁺	ind ⁺ , tyr ⁺
DNA ind ⁺ , tyr ⁻ , 0.01 µg./ml.			
non-heated	3349	0	0
renatured	939	0	0
denatured	14	0	0
DNA ind ⁻ , tyr ⁺ , 0.03 µg./ml.			
non-heated	0	9455	0
renatured	0	2545	0
denatured	0	27	27
Mixture of DNA's: ind ⁺ , tyr ⁻ , 0.01 µg./ml. ind ⁻ , tyr ⁺ , 0.03 µg./ml.			
non-heated	2029	6095	0
renatured separately	789	2245	0
denatured separately	14	29	33
renatured together	869	2455	0
denatured together	11	37	27

Table 2

The effect of renaturation in mixture of ind⁺, tyr⁺ and ind⁻, tyr⁻ DNA's

Recipient: *B. subtilis* ind⁻, tyr⁻.

DNA of donors used in transformation	Number of transformants		
	ind ⁺	tyr ⁺	ind ⁺ , tyr ⁺
DNA ind ⁺ , tyr ⁺ , 0.01 µg./ml.			
non-heated	1670	1260	840
renatured	320	330	120
denatured	19	33	6
Mixture of DNA's: ind ⁺ , tyr ⁺ , 0.01 µg./ml. ind ⁻ , tyr ⁻ , 0.03 µg./ml.			
non-heated	1680	1220	810
renatured separately	250	300	106
denatured separately	1	9	0
renatured together	324	310	130
denatured together	10	12	6

The aim of the next experiment illustrated in Table 2 was to find out whether one or both strands in the DNA molecule are effective in carrying our phenotypically observable transformation. For this purpose a 1:3 mixture of wild type ($\text{ind}^+, \text{tyr}^+$) DNA and $\text{ind}^-, \text{tyr}^-$ DNA, extracted from respective mutants, was denatured and renatured and used in transformation. For control, transformations were also carried with a mixture of separately renatured wild type DNA and $\text{ind}^-, \text{tyr}^-$ DNA as also with mixture of the non-heated DNA's. Both, single and double transformants were scored. As shown in Table 2, the activity of the $\text{ind}^+, \text{tyr}^+$ DNA renatured together with the $\text{ind}^-, \text{tyr}^-$ DNA is not lower than that of the mixture of these DNA's renatured separately.

DISCUSSION

Denaturation and renaturation of $\text{ind}^-, \text{tyr}^+$ with $\text{ind}^+, \text{tyr}^-$ DNA of *Bacillus subtilis* results in production of hybrid DNA, as has been proved by many workers by density gradient centrifugation. The concentration of $\text{ind}^+, \text{tyr}^-$ DNA was 3 times lower than the concentration of the $\text{ind}^-, \text{tyr}^+$ DNA. Thus the probability for any strand of the former DNA to reunion with the respective complementary strand of the genetically different $\text{ind}^-, \text{tyr}^+$ DNA was 3 times higher than for recombining with its own complementary strand. The number of transformants produced by the DNA's renatured in mixture did not differ remarkably from the number of transformants achieved with the DNA's renatured separately and mixed afterwards. Consequently, the hybrid DNA must be biologically active. However, the hybrid DNA seems to carry alternatively one marker like the mixture of DNA's brought together after renaturation or the mixture of non-heated DNA's. This can be simply explained if one assumes that both markers, if present in the same DNA ($\text{ind}^+, \text{tyr}^+$), are represented by respective parts of the same strand. In this case hybridization cannot lead to production of molecules carrying both markers. One may also assume that hybrid DNA carrying both markers is produced, that the markers are parts of complementary strands, but the strands separate before integration into the genome of the recipient cell. Each marker finds its way to a different daughter cell. This supposition seems to be less probable in view of the experiment presented in Table 2.

Results demonstrated in Table 2 are in accordance with the supposition that in transformation only one of the two strands is effective in carrying out a phenotypically observable transformation. If both strands were necessary for phenotypic expression then the efficiency of double transformants produced by a mixture of $\text{ind}^+, \text{tyr}^+$ and an excess of $\text{ind}^-, \text{tyr}^-$ DNA's renatured together should be lower than the efficiency

of doubles obtained with the same DNA's renatured separately and brought together afterwards. This is, however, not the case.

We wish to thank Miss M. Świdorska for her excellent technical assistance in this work.

SUMMARY

By heating and annealing mixtures of DNA from two mutants of *B. subtilis* viz. (1), DNA ind⁺,tyr⁻ and DNA ind⁻,tyr⁺ or (2), DNA ind⁺,tyr⁺ and DNA ind⁻,tyr⁻, hybrid DNA's were obtained.

Results of transformations on *B. subtilis* ind⁻,tyr⁻ recipient cells with hybrid DNA's suggest that both markers are situated on one strand of DNA molecule and that only one of the two strands is effective in carrying out a phenotypically observable transformation.

REFERENCES

- [1] Anagnostopoulos C. & Spizizen J. - *J. Bact.* **81**, 741, 1961.
- [2] Doty P., Marmur J., Eigner J. & Schildkraut C. L. - *Proc. Natl. Acad. Sc.* **46**, 461, 1960.
- [3] Herriott R. M. - *Proc. Natl. Acad. Sc.* **47**, 146, 1961.
- [4] Marmur J. - *J. Mol. Biol.* **3**, 208, 1961.
- [5] Marmur J. & Doty P. - *Nature* **183**, 1427, 1959.
- [6] Marmur J. & Doty P. - *J. Mol. Biol.* **5**, 109, 1962.
- [7] Marmur J. & Lane D. - *Proc. Natl. Acad. Sc.* **46**, 453, 1960.
- [8] Marmur J., Rownd R. & Schildkraut C. L., in *Progress in Nucleic Acid Research* (J. N. Davidson & W. E. Cohn, eds.) vol. I Acad. Press N.Y. 1963.
- [9] Marmur J., Schildkraut C. L. & Doty P. - *J. Chimic Phys.* 945, 1961.
- [10] Schildkraut C. L., Marmur J. & Doty P. - *J. Mol. Biol.* **3**, 595, 1961.
- [11] Spizizen J. - *Proc. Natl. Acad. Sc.* **44**, 1072, 1958.
- [12] Stumpf P. K. - *J. Biol. Chem.* **169**, 260, 1947.

BIOLOGICZNE EFEKTY RENATURACJI MIESZANINY GENETYCZNIE ODMIENNYCH PREPARATÓW DNA Z *BACILLUS SUBTILIS*

Streszczenie

Do transformacji szczepu auktotrofowego *B. subtilis* ind⁻,tyr⁻ niezdolnego do syntezy indolu i tyrozyny użyto hybrydy DNA uzyskane przez renaturację mieszanek: DNA ind⁺,tyr⁻ i DNA ind⁻,tyr⁺, oraz DNA ind⁺,tyr⁺ i DNA ind⁻,tyr⁻.

Wyniki doświadczeń sugerują, iż markery syntezy indolu i tyrozyny umieszczone są u *B. subtilis* na jednej nici DNA, oraz iż dla fenotypowego wyrażenia cechy w transformacji wystarczy jedna nić.

Received 25 November 1963.

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TRYPTOPHAN IN ERYTHROCYTES AND PLASMA OF MAN AND SOME ANIMALS

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This paper is intended as a homage to the memory of the late Professor Doctor Bolesław Skarżyński to whom our Department, founded only nineteen years ago, owes a debt of gratitude for his care, advice and unfailing helpfulness which had helped us to overcome the initial difficulties of our work and encouraged our studies.

The metabolism of tryptophan in the organism proceeds through many pathways. The determination of tryptophan and its metabolites in body fluids may be particularly valuable in diagnosis of certain metabolic disorders.

Few data may be found in the literature on the content of tryptophan in erythrocytes and in plasma. Moreover, the values reported by various authors are divergent, which might have been caused by the different methods applied. So far, the microbiological [3, 4, 13, 5] and semi-quantitative paper chromatography [9] methods have been used. According to the studies of McMenamy [6, 7, 8] and our own results [10] tryptophan is present in the plasma in two forms, free and loosely bound to albumin.

The aim of the present work was to examine the form of tryptophan present in erythrocytes, assess its relation to plasma tryptophan and see whether this relation is similar in man and in animals. The colorimetric method [11] was applied for tryptophan determination, and dialysis for separation of free and bound tryptophan.

MATERIALS AND METHODS

Blood samples. Blood was taken from man, pig, cow, dog and hen after an overnight fast, heparinized and immediately centrifuged at 4° for 20 min. at 2000 r.p.m. Plasma was separated from erythrocytes, the

layer of leucocytes and platelets was discarded, and the erythrocytes were washed once with physiological saline solution.

Determinations of free and bound tryptophan. One ml. of plasma or packed erythrocytes was dialysed in cellophane tubing (Dialysing Puling Fischer, U.S.A.) against 30 ml. of distilled water for 20 hr. at about 4°. Under these conditions, already after 1 hr. the erythrocytes were completely haemolysed.

Free tryptophan was determined in the lyophilized diffusate, and bound tryptophan in the dialysis residue after deproteinization with 20% trichloroacetic acid. Tryptophan was identified by Ehrlich's test on paper chromatograms developed in the following solvent systems: I, isopropanol - conc. ammonia - water (200 : 10 : 20, by vol.); II, sec.-butanol - acetic acid - water (120 : 30 : 50, by vol.). Quantitative determinations were made by the colorimetric method [11], the procedure being as follows. To 1 ml. sample containing 2 - 40 µg. of tryptophan, 2 ml. of conc. acetic acid containing FeCl₃ in 2 mM concentration, and 2 ml. of conc. sulphuric acid were successively added, and stirred; then the colour was estimated in an Unicam spectrophotometer at 545 mµ.

Tryptophan-binding ability of plasma protein and erythrocyte capacity for tryptophan were determined by incubating for 1 hr. at 37° a mixture of 2 ml. of plasma and 2 ml. of packed erythrocytes with 400 µg. of L-tryptophan (British Drug Houses, Ltd.) dissolved in 0.25 ml. of physiological saline solution. After centrifugation, tryptophan was determined in plasma and in erythrocytes as described above.

RESULTS AND DISCUSSION

The content of tryptophan in normal human erythrocytes amounted on the average to 1 mg.%. This value was much higher than those reported by other authors (Table 1). The low values obtained by the chromatographic method could be explained by the losses of tryptophan on chromatography being much greater than those of other amino acids [12]. Lower values obtained by microbiological methods might have been due to the applied drastic methods of deproteinization, e.g. with sodium tungstate and sulphuric acid. Blum [2] has also reported that some indole compounds are oxidized during denaturation of oxyhaemoglobin. Moreover, the losses in tryptophan may be caused by binding of tryptophan to nicotinamide-adenine dinucleotides [1]. The procedure used in the presented experiments permitted to avoid the denaturation of haemoglobin because free tryptophan was determined in the diffusate of erythrocytes.

As may be seen from the results presented in Table 2 the contents of total tryptophan in the plasma of man and some animals were very similar. On the other hand, the ratio of bound to free tryptophan

Table 1

The content of tryptophan in human plasma and erythrocytes according to various workers

Authors	Plasma tryptophan	Erythrocytes tryptophan	Method
Dunn <i>et al.</i> [3]	1.14 mg. %	0.17 mg. %	microbiological
Steele <i>et al.</i> [13]	91 μ moles/kg water (~ 1.8 mg. %)		microbiological
Hier & Bergeim [4]	1.11 mg. %, male 0.98 mg. %, female		microbiological
Johnson & Bergeim [5]	1.27 mg. %	0.29 mg. %	microbiological
McMenamy <i>et al.</i> [9]		21 μ moles/kg. water (~ 0.43 mg. %)	chromatographic
Present results	1.4 mg. %	1.0 mg. %	colorimetric

Table 2

Tryptophan in plasma and erythrocytes of man and some animals

The results are expressed as mg./100 ml. of plasma or packed erythrocytes; they are averages from the indicated number of experiments; in parentheses the limit values are given.

Animal	No. of expts.	Plasma				Erythrocytes	
		Content of tryptophan			Tryptophan-binding ability	Content of tryptophan	Tryptophan capacity
		free	bound	total			
Man	3	0.8 (0.6-1.0)	0.6 (0.5-0.8)	1.4	3.1 (2.6-3.6)	1.0 (0.6-1.5)	2.7 (2.6-2.8)
Pig	4	0.7 (0.4-0.8)	0.7 (0.5-0.8)	1.4	2.5 (2.1-2.9)	1.3 (0.8-1.6)	1.8 (1.6-2.0)
Cow	3	0.9 (0.7-1.0)	0.5 (0.4-0.5)	1.4	1.6 (1.1-2.2)	1.3 (1.2-1.5)	2.3 (2.0-2.5)
Dog	2	1.4 (1.0-1.7)	0.8 (0.5-1.1)	2.2	1.7 (1.1-2.2)	1.0 (0.7-1.2)	1.4 (1.2-1.5)
Hen	3	1.3 (1.0-1.7)	0.4 (0.3-0.5)	1.7	1.1 (0.8-1.3)	3.8 (3.2-4.5)	5.2 (4.5-5.8)

(Table 3) varied rather widely in the examined animals from 0.3 in the hen to 1.0 in the pig. Tryptophan-binding ability of the plasma was the highest in man (3.1 mg. %) and the lowest in the hen (1.1 mg. %). In hen plasma the comparatively small content of bound tryptophan and lower

Table 3

Ratios of concentrations of tryptophan in erythrocytes to its concentrations in plasma

Animal	Ratio of bound to free Try in plasma	Ratio of total Try in erythrocytes to Try in plasma
Man	0.75	0.72
Pig	1.00	0.93
Cow	0.56	0.93
Dog	0.57	0.46
Hen	0.31	2.24

binding ability for this amino acid seem to be connected with lower albumin content found in bird plasma.

In erythrocytes, tryptophan was present only in the free form and no tryptophan bound to protein was found. The contents of free tryptophan varied in mammals from 1.0 to 1.3 mg.‰ and were much higher (3.8 mg.‰) in the hen (Table 2). The capacity of erythrocytes for tryptophan was also several times higher in the hen than in mammals. These results seem to indicate increased metabolic requirement for tryptophan in hen erythrocytes. This may be connected with the comparatively high content of nicotinamide-adenine dinucleotides and of nicotinic acid in nucleated erythrocytes and it seems probable that tryptophan participates in the synthesis of these compounds.

SUMMARY

The content of free and bound tryptophan in plasma and erythrocytes of man and some animals as well as the tryptophan-binding ability of plasma and tryptophan capacity of erythrocytes were studied, dialysis and the colorimetric method being applied for direct determination of tryptophan. Differences in the content of, and capacity for, tryptophan were found between nucleated and non-nucleated erythrocytes.

REFERENCES

- [1] Alivisatos S. G. A., Ungar F., Jibril A. & Mourkides G. A. - *Biochim. Biophys. Acta* **51**, 361, 1961.
- [2] Blum J. J. & Ling N. S. - *Biochem. J.* **73**, 530, 1959.
- [3] Dunn M. S., Schott H. F., Frankl W. & Rockland L. B. - *J. Biol. Chem.* **157** 387, 1945.
- [4] Hier S. W. & Bergeim O. - *J. Biol. Chem.* **163**, 129, 1946.
- [5] Johnson C. A. & Bergeim O. - *J. Biol. Chem.* **188**, 833, 1951.

- [6] McMenamy R. H., Lund C. C. & Oncley J. L. - *J. Clin. Invest.* **36**, 1672, 1957.
- [7] McMenamy R. H. & Oncley J. L. - *J. Biol. Chem.* **233**, 1436, 1958.
- [8] McMenamy R. H., Lund C. C., Van Marcke J. & Oncley J. L. - *Arch. Biochem. Biophys.* **93**, 135, 1961.
- [9] McMenamy R. H., Lund C. C., Neville G. J. & Wallach D. F. H. - *J. Clin. Invest.* **39**, 1675, 1960.
- [10] Opieńska-Blauth J., Charęziński M. & Brzuszkiewicz H. - *Clin. Chim. Acta* **8** 260, 1963.
- [11] Opieńska-Blauth J., Charęziński M. & Berbeć H. - *Anal. Biochem.* **6**, 69, 1963.
- [12] Opieńska-Blauth J., Charęziński M., Sanecka M. & Brzuszkiewicz H. - *J. Chromat.* **7**, 321, 1962.
- [13] Steele B. F., Reynolds M. S. & Baumann C. A. - *J. Nutrition* **40**, 145, 1950.

TRYPTOFAN W KRWINKACH I OSOCZU CZŁOWIEKA I NIEKTÓRYCH ZWIERZĄT

Streszczenie

Przy zastosowaniu dializy i metody kolorymetrycznej dla bezpośredniego oznaczenia tryptofanu przeprowadzono badania nad zawartością tryptofanu wolnego i związanego w osoczu człowieka i różnych zwierząt. Ponadto określono zawartość tryptofanu w krwinkach u tych samych zwierząt.

Równolegle przeprowadzono oznaczenia zdolności wiążącej tryptofan w osoczu i pojemności tryptofanowej w krwinkach. Stwierdzono wyraźne różnice zarówno pod względem zawartości tryptofanu jak i pojemności tryptofanowej między krwinkami jądrzastymi (u kury) a krwinkami ssaków.

Received 28 November 1963.

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INCORPORATION OF ^{32}P INTO NUCLEIC ACIDS AND PHOSPHOLIPIDS OF THE LIVERS OF TUMOUR-BEARING RATS

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As it has been shown by Kelly & Jones [7], Payne *et al.* [12], Tyner *et al.* [14], Way *et al.* [15] and Griffin [5], the presence of a tumour in mice or rats markedly stimulates the incorporation of radioactive precursors of nucleic acids in the liver. It seemed worthwhile to study the changes in the incorporation in relation to the age of the grafted tumour. The next step, will be an autoradiographic study of the response of the different types of liver cells (hepatic, Kupffer, and duct cells).

The work here reported concerns the effect of the grafted tumour on the incorporation of ^{32}P -labelled phosphate into nucleic acids and into other fractions of the liver during 3 weeks following implantation of the tumour.

Guérin's T8 tumour was used; this is a uterine epithelioma isolated by Guérin in a rat in 1934 [6] which has since been continuously transplanted in the rat. It is a highly malignant tumour which produces a great number of lymph node metastases. During the first week following implantation the tumour does not develop, on the contrary, the volume of the graft decreases; some tumour cells, however, already begin to invade the lymph nodes. During the second week, the tumour and the metastases develop rapidly. This rapid development continues for one or two weeks and is accompanied by necrosis of the central part of the tumour; the death of the animal occurs about four weeks after implantation.

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MATERIAL AND METHODS

Twelve 6-month-old Wistar rats, weighing approximately 200 g. each, were used. Six were grafted under the skin on the right side of the back with the Guérin tumour. The animals were killed 8, 15, and 21 days after implantation. Two grafted and two control animals each received an injection of ^{32}P -labelled disodium phosphate ($1\text{ }\mu\text{c/g. body wt.}$). Disodium phosphate furnished by the Centre d'Etudes Nucléaires, Saclay, France, was used. The animals were starved for 12 hr. before the injection, further starved for 18 hr. (only given water *ad libitum*), then killed by decapitation. The livers were removed and frozen immediately on dry ice.

Each liver was ground in 50 ml. of physiological saline solution and treated according to the Schmidt & Thannhauser method [13] as modified by MacIndoe & Davidson [8] in order to obtain the separation of the different phosphorus-containing fractions. The acid-soluble fraction was extracted with cold 5% trichloroacetic acid and contained mainly the precursors of the nucleic acids. The lipids were extracted by successive treatment with cold and warm alcohol, acetone, ether and chloroform, to assure complete removal of the highly radioactive phospholipids. The residue was treated overnight with 1M-sodium hydroxide at 37° ; the supernatant was then neutralized, and the addition of a 30% trichloroacetic acid solution resulted in the separation of a soluble fraction containing RNA, and a precipitate containing DNA. In the RNA fraction, some inorganic phosphorus was present; this was precipitated according to Mathison [10]. The DNA fraction contained some protein which, however, had no phosphorus and so did not interfere with radioactivity measurements.

In each fraction, phosphorus was determined by the Brigg's colorimetric method [2, 3, 9]; each estimation was made in duplicate. The radioactivity was measured with a flow counter of "Tracelab" type.

RESULTS

The results presented in Table 1 and in Fig. 1 indicate that the radioactivity of the phosphorus in the different fractions isolated from the liver varies with the age of the tumour, and that the incorporation of ^{32}P into DNA of the liver is increased in tumour-bearing animals, as compared to the control animals. The incorporation reached a maximum on the 15th day, then decreased, but on the 21st day still remained greater than the control value.

The increase in the radioactivity of the RNA fraction was less marked than that of the DNA fraction. The maximum for the RNA fraction

was observed on the 8th day, a similar value on the 15th day, and a much lower one on the 21st day. The incorporation pattern of ^{32}P into the acid-soluble fraction was fairly similar to that of the DNA but the increase was less marked. The incorporation of ^{32}P into phospholipids presented a different pattern, the incorporation being markedly increased

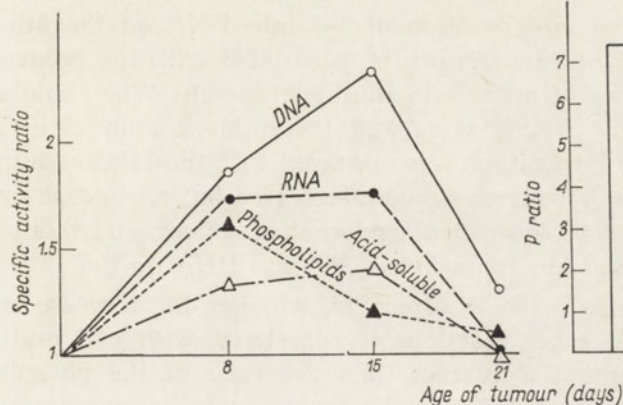


Fig. 1

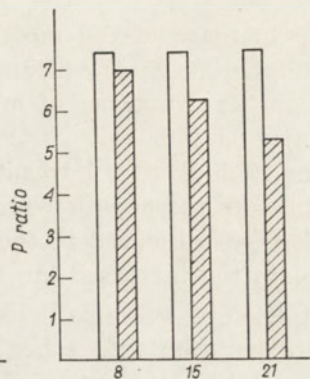


Fig. 2

Fig. 1. Incorporation of ^{32}P -labelled phosphate in the liver of tumour-bearing rats. Ratio of specific activity was calculated as counts/min./mg.P in liver fractions of tumour-bearing animals, per counts/min./mg.P in liver fractions of controls.

(O), DNA; (●), RNA; (Δ), acid-soluble fraction; (▲), phospholipids.

Fig. 2. Ratio of phosphorus in phospholipids to phosphorus in DNA, in the liver of tumour-bearing and control rats. Hatched columns, ratio for tumour-bearing animals; unhatched columns, ratio for control animals.

Table 1

The incorporation of ^{32}P -labelled phosphate into phosphorus compounds in the liver of tumour-bearing and control rats

Results are expressed as counts $\times 10^{-4}$ /min./mg. P and as ratio of specific activity in tumour-bearing and control rats.

Age of tumour...	8 days			15 days			21 days		
	Tumour-bearing	Control	Spec. act. ratio	Tumour-bearing	Control	Spec. act. ratio	Tumour-bearing	Control	Spec. act. ratio
Phospholipids	28.0	17.4	1.61	25.0	20.8	1.2	21.0	19.3	1.09
Acid-soluble fraction	22.3	16.8	1.33	24.6	17.4	1.41	17.0	16.8	1.01
DNA	1.68	0.9	1.86	2.11	0.9	2.34	0.93	0.71	1.31
RNA	12.1	7.0	1.73	12.0	6.8	1.76	7.9	7.8	1.01

on the 8th day after implantation of the tumour and much lower on the 15th day, while in the other fractions at that time a further increase was observed or the values remained on a similarly high level.

DISCUSSION

The increase of the incorporation of ^{32}P into DNA on the 8th day after implantation of the tumour, can be correlated with the occurrence of a significant number of mitoses in the hepatic cells. When colchicine was injected into rats at this stage and the mitoses counted in liver sections 15 days after implantation, it appeared that the relative number of mitoses in the hepatic cells was much decreased but an intense multiplication was observed in the reticuloendothelial system, and this seems to explain the increased incorporation of ^{32}P into DNA on the 15th day.

An attempt was also made to determine whether the increase in the ^{32}P incorporation into other fractions is correlated with cell multiplication. If this assumption is correct then the ratio of the phosphorus of the acid-soluble fraction, RNA and phospholipids, to phosphorus of DNA used as a standard of reference, should be constant. This method of expressing biochemical results by calculating the amounts of components per cell, was first adopted in 1950 by Davidson & Leslie [4] and is based on the finding of Boivin, Vendrely & Vendrely [1] that the amount of DNA in nuclei of somatic cells from animals of a single species is constant.

Table 2

Ratio of phosphorus of RNA, acid-soluble fraction and phospholipids to DNA phosphorus in the liver of tumour-bearing and control rats

The ratio is calculated as mg. phosphorus in the compound to mg. DNA phosphorus.

Age of tumour...	Control	8 days	15 days	21 days
RNA	5.59	5.48	5.89	5.50
Acid-soluble fraction	6.0	5.52	5.29	4.78
Phospholipids	7.4	6.93	6.25	5.23

The results presented in Table 2 indicate that the ratios of phosphorus of RNA and of the acid-soluble fraction to DNA phosphorus were practically constant, but that for phospholipids decreased gradually (Fig. 2) even on the 8th day, that is at the time when the incorporation of ^{32}P into phospholipids was increased. This indicates that simultaneously with an increase in the synthesis of phospholipids, there is a still more important catabolism resulting in a definite decrease in the amount of phospholipids.

The most marked increase in the incorporation of ^{32}P into all the fractions was observed on the 8th day of tumour growth (Table 1, Fig. 1), that is simultaneously with the multiplication of the hepatic cells, but it is not clear in which way the two phenomena are correlated. It would be very interesting to determine at which stage of the mitotic cycle the change in phospholipid metabolism does occur.

Matsumoto & Hori [11] have shown by histochemical techniques a gradual decrease of the phospholipid content in the livers of mice after sarcoma implantation. This decrease began in the centrolobular region and progressively extended to nearly all the lobule. It was due to destruction of mitochondria and ergastoplasmic membranes. The presented results obtained by biochemical techniques are in agreement with those of Matsumoto & Hori, indicating in addition that increased synthesis does occur but cannot counterbalance the destruction.

SUMMARY

Incorporation of ^{32}P -labelled phosphate into different liver fractions was studied in rats bearing the Guérin's T8 tumours at different stages of development. A marked increase of the incorporation into DNA, RNA and the acid-soluble fraction was observed and can be explained by mitosis of the liver cells. The metabolism of the phospholipids was more complex; their total amount per cell decreased even at the time when the incorporation of ^{32}P increased.

REFERENCES

- [1] Boivin A., Vendrely R. & Vendrely C. - *C. R. Acad. Sci.* **226**, 1061, 1948.
- [2] Briggs A. P. - *J. Biol. Chem.* **53**, 13, 1922.
- [3] Briggs A. P. - *J. Biol. Chem.* **59**, 255, 1924.
- [4] Davidson J. N. & Leslie I. - *Nature* **165**, 49, 1950.
- [5] Griffin A. C. - *Texas Rep. Biol. Med.* **15**, 161, 1957.
- [6] Guérin M. P. - *Bull. Assoc. Franc. Et. Cancer* **23**, 632, 1934.
- [7] Kelly L. S. & Jones H. B. - *Science* **111**, 333, 1950.
- [8] MacIndoe N. M. & Davidson J. N. - *Brit. J. Cancer* **6**, 200, 1952.
- [9] Martland M. & Robison R. - *Biochem. J.* **20**, 847, 1926.
- [10] Mathison G. C. - *Biochem. J.* **4**, 233, 1909.
- [11] Matsumoto T. & Hori S. H. - *Gann* **50**, 141, 1959.
- [12] Payne A. H., Kelly L. S. & White M. R. - *Cancer Res.* **12**, 65, 1952.
- [13] Schmidt G. & Thannhauser S. J. - *J. Biol. Chem.* **161**, 83, 1945.
- [14] Tyner E. P., Heidelberger C. & LePage G. A. - *Cancer Res.* **13**, 186, 1953.
- [15] Way J. L., Mandel H. G. & Smith P. G. - *Cancer Res.* **14**, 812, 1954.

INKORPORACJA ^{32}P W KWASY NUKLEINOWE I FOSFOLIPIDY WĄTROBY U SZCZURÓW Z PRZESZCZEPIONYM NOWOTWOREM

Streszczenie

Badano inkorporację fosforanu znakowanego ^{32}P w DNA, RNA i frakcję kwaso-rozpuszczalną wątroby szczura z przeszczepionym epithelioma Guérin'a T8, w różnych stadiach jego rozwoju. Stwierdzono zwiększenie inkorporacji, które mogło wynikać z szybkiego podziału komórek wątrobowych. Przemiana fosfolipidów była bardziej złożona; ich całkowita zawartość w komórce malała nawet w okresie, gdy inkorporacja ^{32}P wzrastała.

Received 3 December 1963

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DECOMPOSITION OF RIBONUCLEIC ACID FROM THE LIVER MICROSOMAL FRACTION

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In the previous paper [4] it has been demonstrated that *Escherichia coli* ribosomes incubated in ammonium formate buffer with low magnesium content, released into the medium free bases and nucleosides deaminated at position 6. This release seems to be connected not only with RNase, which is known to be present in the ribosomes [5], but also with nucleosidase and deaminase activities. It has been suggested that this process is associated with the decomposition of messenger RNA (mRNA) bound to ribosomes [1]. If this is really so, then the release of deaminated bases and nucleosides from ribosomes should be a more widely occurring phenomenon.

In the present work, free bases and nucleosides arising on decomposition of RNA, were isolated from the microsomal fraction of guinea pig liver and identified.

EXPERIMENTAL

Special reagents. Tris was obtained from Sigma Co. (St. Louis, U.S.A.); DEAE-cellulose from Carl Schleicher & Schuell Co. (U.S.A.); adenine, guanine, cytosine and thymidine from Dr. T. Schuchardt, B.M.B.H. (München); adenosine and guanosine from Reanal (Budapest, Hungary); cytidine from California Corp. for Biochem. Research (U.S.A.); hypoxanthine and uridine from L. Light (England); inosine from P.A.B.S.T. Laboratory (U.S.A.); thymine from Fluka A. G., Buchs S. G. (Switzerland); uracil from Fabryka Odczynników Chemicznych (Gliwice).

Methods. Protein was determined colorimetrically according to Lowry *et al.* [3]. RNA in ribosomes was estimated after hydrolysis in 0.5 M-KOH at 37° for 20 hr.

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Decomposition of RNA from the microsomal fraction

Three guinea pigs were killed by decapitation, the livers were excised and the microsomal fraction was obtained by centrifugation between 18 000 and 100 000 *g*, model Omicron (West Germany) centrifuge being used. The medium contained saccharose, KCl, KHCO₃, MgCl₂, and tris-HCl according to Lingrel & Webster [2]. The obtained microsomal fraction was washed with 0.25 M-saccharose - 0.001 M-magnesium acetate solution, and incubated in 0.005 M-ammonium formate buffer of pH 6.2. From the incubation mixture, samples were taken at appropriate time intervals. To each sample 2 vol. of absolute ethanol were added and the formed sediment was centrifuged off and discarded. In the supernatant fluid the RNA degradation products were determined by measuring the absorption at 260 m μ in a Unicam SP 500 spectrophotometer.

The results shown in Fig. 1 indicate that during incubation some substances soluble in 66% ethanol and absorbing at 260 m μ are released from the microsomes.

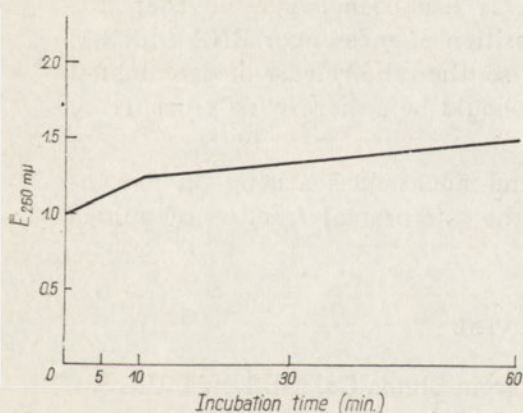


Fig. 1

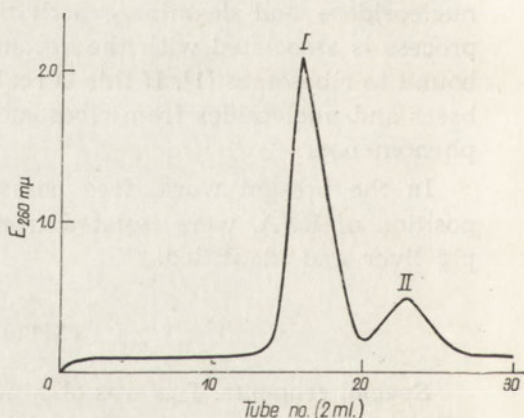


Fig. 2

Fig. 1. Decomposition of RNA of the microsomal fraction. Microsomal fraction (350 mg. protein) was incubated in 14 ml. of 0.005 M-ammonium formate buffer, pH 6.2, for 1 hr. at 37°. From the incubation mixture, 2.7 ml. samples were taken, added with 2 vol. of absolute ethanol and centrifuged at 2°. In the supernatant fluid the extinction was estimated at 260 m μ .

Fig. 2. Separation of RNA decomposition products on a DEAE-cellulose column. The microsomal fraction was incubated as described in the legend to Fig. 1. Incubation was stopped by adding 2 vol. of absolute ethanol. After centrifugation at 2° the supernatant fluid was evaporated and the residue was dissolved in water, defatted, and separated on a DEAE-cellulose column (1.2 \times 20 cm.) in the same buffer as used for the incubation.

A similar experiment was performed on ribosomes obtained from guinea pig liver by 5% sodium deoxycholate. The ribosomes were washed with 0.25 M-saccharose - 0.001 M-MgCl₂ solution buffered with tris-HCl to pH 7.8; then the amount of RNA was estimated and the ribosomes were incubated in the same saccharose - MgCl₂ solution. The amount of UV-absorbing substances released during 1 hr. incubation corresponded to decomposition of about 0.5% RNA present in the ribosomes before incubation.

Identification of RNA decomposition products

The microsomal fraction, 0.5 - 1.0 g. protein, was incubated for 60 min. in 0.005 M-ammonium formate buffer, pH 6.2, added with ethanol and centrifuged; the supernatant fluid was evaporated in a desiccator over calcium chloride and the residue was dissolved in about 2 ml. water. It was then defatted with a mixture of alcohol and ether (1:4, v/v) and fractionated with 0.005 M-ammonium formate buffer, pH 6.2, on a DEAE-cellulose column equilibrated previously with the same buffer (Fig. 2).

Under these conditions, nucleosides and bases were eluted from the column. They separated into two fractions which were then evaporated *in vacuo*. As fraction I was contaminated with saccharose, this was removed by paper electrophoresis in acid and then in alkaline medium.

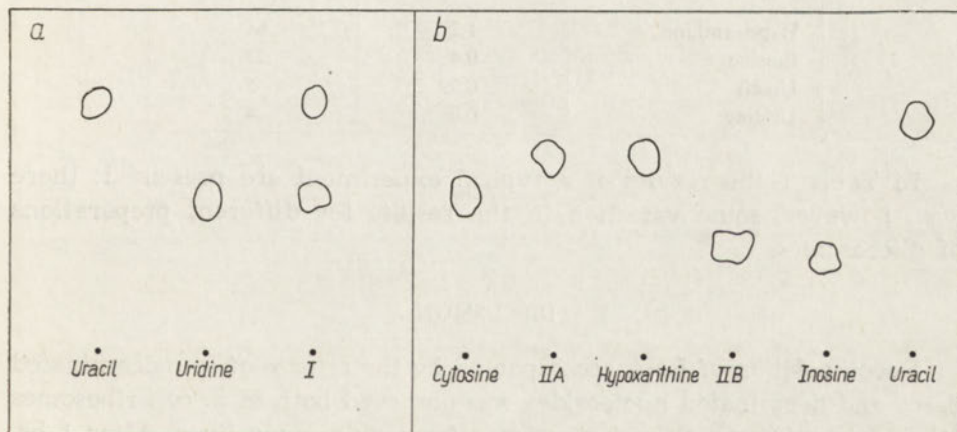


Fig. 3. Chromatograms of decomposition products of microsomal RNA. (a), Fraction I from the DEAE-cellulose column after electrophoresis at pH 1.3 and 9.2; (b), fractions IIA and IIB after electrophoresis at pH 9.2.

The electrophoresis with 0.5 M-formic acid, pH 1.3, was carried out on Whatman no. 3 paper at 44 v/cm. for 2 hr. The UV-absorbing substances moving toward the cathode were eluted with water and submitted to electrophoresis in 0.05 M-sodium tetraborate, pH 9.2, the time and potential applied being the same as for electrophoresis in acid medium. The

band moving toward the anode was eluted with water and chromatographed on Whatman no. 1 paper in a solvent of butanol - acetic acid - water (4:1:5, by vol.).

Fraction II from the DEAE-cellulose column contained no saccharose and was directly submitted to electrophoresis in sodium tetraborate. The two UV-absorbing bands obtained, IIA and IIB, were eluted and chromatographed as described above (Fig. 3).

From the chromatograms the UV-absorbing areas were eluted with water and absorption spectra were taken at pH 2, 7 and 12. On the basis of the R_F values and the absorption spectra, uracil and uridine were identified in fraction I and hypoxanthine and inosine in fraction II. The amounts of the identified compounds were calculated from the molecular extinction coefficients.

Table 1

Bases and nucleosides released from the microsomal fraction of guinea pig liver

The results are expressed as μ moles of bases and nucleosides released from an amount of microsomes corresponding to 500 mg. protein.

Compound	μ moles	%
Hypoxanthine	1.2	68
Inosine	0.4	23
Uracil	0.09	5
Uridine	0.08	4

In Table 1 the results of a typical experiment are presented; there was, however, some variation in the results for different preparations of microsomes.

DISCUSSION

Decomposition of RNA accompanied by the release of free deaminated bases and deaminated nucleosides was observed both in *E. coli* ribosomes [4] and in microsomes and ribosomes from guinea pig liver. After 1 hr. incubation this decomposition in *E. coli* did not exceed 1% of the RNA present in the ribosomes, and in guinea pig ribosomes it amounted to 0.5%. Similar processes were also observed in other organisms. Hahn & Wolfe [1] have found uracil, guanine, adenine and hypoxanthine, and only slight amounts of nucleotides in a culture medium of *Bacillus cereus*.

It seems permissible to suggest that the process through which the deaminated RNA decomposition products become released from the ribosomes, may be related to protein biosynthesis. A tentative suggestion

may be perhaps advanced to explain one of the main steps of protein synthesis, namely the mechanism of circulation of sRNA between ribosomes and the soluble cell fraction.

According to the present views, sRNA to which an amino acid is joined, combines with mRNA on the ribosomes where the polymerization of amino acids takes place; then the completed protein molecule is released and the free sRNA returns to the soluble cell fraction and combines again with amino acids. The mechanism of release of sRNA from the ribosome is unknown. If according to Crick's adaptor theory sRNA is bound by hydrogen bonds to triplets of mRNA, then the deamination of one of the three bases of mRNA would permit the release of sRNA. Partly deaminated mRNA, no longer able to combine with sRNA, could be susceptible to the action of RNase [6] and then to nucleosidase, being decomposed to deaminated free bases. This mechanism seems to be in agreement with the suggestions of Tal & Elson [5] on the participation of RNase in the biosynthesis of a protein molecule. It would also offer an explanation of the instability of mRNA and the necessity for its continual resynthesis.

This work was supported in part by a grant from the United States Department of Agriculture, Agricultural Research Service.

SUMMARY

Microsomes and ribosomes from guinea pig liver incubated in a medium with low or sufficient magnesium content, release deaminated nitrogen bases and corresponding nucleosides. Hypoxanthine, inosine, uracil and uridine were identified.

It has been suggested that the released bases and nucleosides are mRNA decomposition products; this could imply a relation between this process and protein biosynthesis.

REFERENCES

- [1] Hahn F. E. & Wolfe A. D. - *Biochem. Biophys. Res. Comm.* **6**, 464, 1962.
- [2] Lingrel J. B. & Webster G. - *Biochim. Biophys. Acta* **61**, 942, 1962.
- [3] Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. - *J. Biol. Chem.* **193**, 267, 1951.
- [4] Szafranski P. & Lane B. G. - *Biochim. Biophys. Acta* **61**, 141, 1962.
- [5] Tal M. & Elson D. - *Biochim. Biophys. Acta* **76**, 40, 1963.
- [6] Vandendriessche L. - *C. R. Trav. Lab. Carlsberg Ser. Chim.* **27**, 342, 1951.

ROZKŁAD RNA FRAKCJI MIKROSOMOWEJ WĄTROBY

Streszczenie

Wykazano, że z frakcji mikrosomowej, a także z rybosomów wątroby świnki morskiej, inkubowanych w środowisku z obniżoną lub dostateczną zawartością jonów Mg^{2+} , uwalniają się zdeaminowane zasady azotowe i odpowiednie nukleozydy. Zidentyfikowano hipoksantynę, inozynę, uracyl i urydynę.

Istnieją przypuszczenia, że uwalniające się zasady i nukleozydy pochodzą z rozkładu informacyjnego RNA, co mogłoby wskazywać na powiązanie tego procesu z biosyntezą białka.

Received 4 December 1963.

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COMPARATIVE STUDIES ON GLUTAMINE CONTENT IN ANIMAL TISSUES

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Glutamine is known to play an important role in biological processes of living organisms. Its significance as a source of ammonia in the kidneys of ureothelic animals is well established [9, 8]. The role of glutamate-glutamine system in the ammonia-binding process in the brain seems to be important for the activity of the nervous tissue [3]. There are many biosynthetic reactions in which glutamine takes part, e.g. purine synthesis [1]. Nevertheless, the data concerning glutamine content in animal tissues, especially in the tissues of lower animals, are scarce.

In this paper some data on glutamine content in the tissues of 17 animal species are presented.

MATERIALS AND METHODS

Animals and tissues. The animals were either laboratory-bred or were caught in their natural environment. They were killed, usually by decapitation, and the tissues to be examined were dipped into 15% trichloroacetic acid as soon as possible, and homogenized in the acid; in the extract, glutamine was estimated. The earthworms were kept for a few days preceding the experiment in a beaker filled with wet filter paper, in order to clean the digestive tract and to facilitate the separation of muscle. The human striated muscle was obtained from the Surgical Clinic and was taken *intra operationem*.

Glutamine determination. This was made by the enzymic method described previously [11]. Glutamine was decomposed by rat kidney glutamine amidohydrolase preparation, and ammonia estimated with the microdiffusion technique.

RESULTS AND DISCUSSION

Table 1 presents the content of glutamine in the muscles of all species tested, in the kidneys of vertebrates, in the gills of fishes and lamprey, and in the blood of some animals. Glutamine content in the

Table 1

Glutamine content in the trichloroacetic acid extract of animal tissues

The tissues were homogenized in 15% trichloroacetic acid, the extracts neutralized and the content of glutamine was estimated by the enzymic method [11]. Mean values, \pm S.D. where applicable, are given; the numbers of animals tested (or the numbers of pooled portions of tissue from several small animals) are given in parentheses.

Animal	Glutamine (μ mole/1 g. wet wt.)			
	Muscle	Kidney	Blood	Gills
Mammalia				
Man	16.8 (2)	—	0.77 \pm 0.15 (12)	
Rat, <i>Rattus norvegicus</i>	4.0 \pm 0.79 (5)	6.0 \pm 2.5 (6)	1.7 \pm 0.75 (8)	
Guinea pig, <i>Cavia porcellus</i>	3.4 \pm 1.65 (4)	4.0 \pm 2.8 (4)	1.3 \pm 0.02 (4)	
Rabbit, <i>Oryctolagus cuniculus</i>	8.1 \pm 2.1 (3)	4.0 \pm 0.7 (4)	2.2 \pm 0.8 (4)	
Aves				
Pigeon, <i>Columba livia</i>	3.7 \pm 1.1 (4)	2.7 \pm 0.9 (5)	4.0 \pm 1.5 (4)	
Reptilia				
Turtle, <i>Testudo graeca</i>	6.8 (2)	4.9 (2)	1.4 (2)	
Amphibia				
Frog, <i>Rana esculenta</i>	7.5 \pm 0.82 (3)	9.7 \pm 3.3 (3)	4.4 \pm 2.5 (3)	
Pisces				
Carp, <i>Cyprinus carpio</i>	1.1 (2)	1.3 (2)	—	1.1 (1)
Trout, <i>Salmo irideus</i>				
Gibbons	2.5 (2)	2.7 (2)	—	2.3 (2)
Tench, <i>Tinca tinca</i>	0.6 (2)	1.3 (2)	—	0.7 \pm 0.29 (3)
Vimba, <i>Vimba vimba</i>	1.3 (2)	1.6 (2)	—	0.6 \pm 0.12 (3)
Eel, <i>Anguilla anguilla</i>	1.9 \pm 0.2 (4)	2.6 \pm 1.5 (5)	1.0 \pm 0.2 (4)	2.0 \pm 0.6 (4)
Cyclostomata				
Lamprey, <i>Lamperta fluviatilis</i>	1.2 (3)	2.0 (2)	0.4 (2)	1.0 (3)
Crustacea				
Crawfish, <i>Cambarus affinis</i>	10.2 \pm 1.7 (6)	—	—	—
Molusca				
Clam, <i>Unio pictorum</i>				
adductor	5.9 \pm 1.1 (3)	—	—	—
foot	5.0 \pm 1.1 (4)	—	—	—
Snail, <i>Helix pomatia</i>	0.6 \pm 0.19 (5)	—	—	—
Annelida				
Earthworm, <i>Lumbricus terrestris</i>	9.5 \pm 4.3 (6)	—	—	—

kidneys of fishes appeared to be lower than in the kidneys of mammals and frog. This may be consistent with the suggestion [7, 6] that AMP in fishes, and glutamine in higher animals are the main sources of urine ammonia. Also the gills of fishes through which the majority of waste nitrogen is excreted are poor in glutamine. It has been shown by Makarewicz [6] that the gills have low glutamine amidohydrolase activity.

Among the bloods examined those of frog and pigeon are the richest in glutamine. The content of glutamine in human blood is rather low as compared with other animals.

Although it is a well known fact that muscles of mammals contain free glutamine in a relatively high concentration [2, 4, 10] the biological significance of this compound in muscle metabolism is still obscure. The muscles of fishes seem to contain less glutamine than the muscles of amphibians, birds and mammals. However, the concentration of glutamine in the muscles of all invertebrates tested, except the snail, is unexpectedly high, being the highest in the crawfish and earthworm. Levenbook & Kuhn [5] have shown that *Prodenia eridania* has a considerable glutamine synthetase activity which varies during the development of the insect. These facts and our present results may indicate that glutamine is an important tissue constituent and may play an important role in tissue metabolism not only in higher animals but also in lower animal organisms.

SUMMARY

The content of free glutamine in the tissues of 17 vertebrate and invertebrate animals was estimated by the enzymic method. High glutamine content has been found in the muscles of some invertebrates (crawfish and earthworm) and in human muscle as compared with those of other species.

REFERENCES

- [1] Buchanan J. M. in *The Nucleic Acids* (Eds. E. Chargaff & J. N. Davidson) vol. III, p. 304. Academic Press, New York, London, 1960.
- [2] Hamilton P. B. - *J. Biol. Chem.* **158**, 397, 1945.
- [3] McIlwain H., *Biochemistry and the Central Nervous System*, p. 111, J. & A. Churchill Ltd., London 1959.
- [4] Krebs H. A., Eggleston L. V. & Hems R. - *Biochem. J.* **44**, 159, 1949.
- [5] Levenbook L. & Kuhn J. - *Biochim. Biophys. Acta* **65**, 219, 1962.
- [6] Makarewicz W. - *Acta Biochim. Polon.* **10**, 363, 1963.
- [7] Makarewicz W. & Żydowo M. - *Comp. Biochem. Physiol.* **6**, 269, 1962.
- [8] Rector F. C., Seldin D. W. & Copenhaver J. H. - *J. Clin. Invest.* **34**, 20, 1955.
- [9] van Slyke D. D., Phillips R. A., Hamilton P. B., Archibald R. M., Fitcher P. H. & Hiller A. - *J. Biol. Chem.* **150**, 481, 1943.
- [10] Wu C. - *J. Biol. Chem.* **207**, 775, 1954.
- [11] Żydowo M. & Purzycka J. - *Acta Biochim. Polon.* **10**, 293, 1963.

PORÓWNAWCZE BADANIA ZAWARTOŚCI GLUTAMINY W TKANKACH ZWIERZĄT

Streszczenie

Oznaczono metodą enzymatyczną zawartość wolnej glutaminy w tkankach 17 zwierząt kręgowych i bezkręgowych. W mięśniach raka, dżdżownicy i człowieka znaleziono stężenie glutaminy wyższe niż w tkankach innych zwierząt.

Received 5 December 1963.

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THE FORMS OF PHOSPHORYLATED THIAMINE IN THE MILKS OF DIFFERENT ANIMALS*

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The vitamin B₁ activity of natural materials may stem from that of free thiamine or of a mixture of free thiamine and thiamine phosphates (Fig. 1).

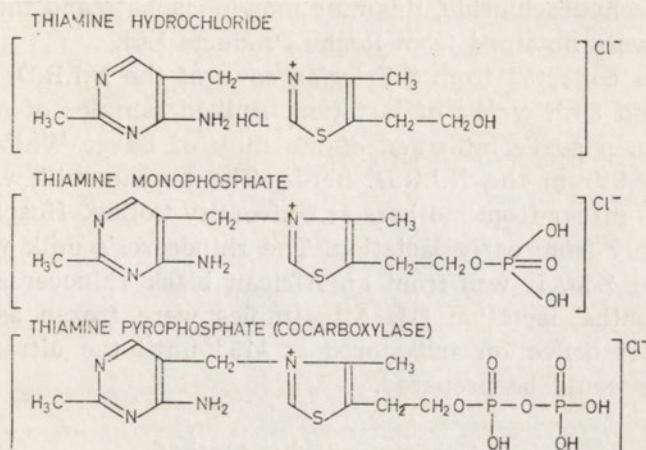


Fig. 1. Formulas of thiamine and of its phosphates.

During early work at this Institute on the vitamin B₁ activity of cow's milk, Houston, Kon & Thompson [11] found that both free and phosphorylated thiamine were present. They showed that the phosphorylated form occurred in large amounts in colostrum or milk early in lactation, but only in trace amounts in mid or late lactation. Further, there was a negative correlation between the content of phosphorylated thiamine and the alkaline phosphomonoesterase titre of the milk. At that time, Houston *et al.* [11] assumed that the phosphorylated form was

* Read in part by M. E. Gregory at the 5th International Congress on Nutrition, Washington, 1960. (Abstracts, p. 14, no. 61).

thiamine pyrophosphate (cocarboxylase), the coenzyme form of the vitamin which predominates in animal tissues. However, shortly afterwards de Jong [12] in Holland reported that he could find no cocarboxylase activity in cow's milk and suggested that the phosphorylated form was more likely to be thiamine monophosphate. Unfortunately de Jong died in a concentration camp before he completed his work and no further investigations were made until recently, when with the newer techniques of paper chromatography and bioautography we were able to demonstrate at this Institute the presence of thiamine monophosphate in cow's milk. The methods used to separate and identify the different vitamin B₁ active compounds present in cow's milk and in the milks of certain other species are now described.

EXPERIMENTAL

Materials

Thiamine hydrochloride, thiamine monophosphate and thiamine pyrophosphate were obtained from Roche Products Ltd.

Milk was collected from 3 Friesian cows of the N.I.R.D. herd, in the 2nd, 21st and 39th weeks of lactation. Bulk samples of mid-lactation British Saanen goat's milk and of the milk of Large White sows were also obtained from the N.I.R.D. herds. The human milk was collected by Dr. M. Gunther from mothers at University College Hospital, London. It was mainly from early lactation. The rhinoceros's milk was obtained from Bristol Zoo. It was from an African black rhinoceros at the end of a 19 months' lactation [1]. All samples were frozen as quickly as possible after collection and stored at -15° until the ultrafiltrates and concentrates could be prepared.

Chromatographic method

Extraction of vitamin B₁ activity from milk. A protein-free milk serum was prepared by ultrafiltering the raw whole milks through a cellophane membrane under reduced pressure as described by Gregory [9]. The ultrafiltrate was cooled and mixed well with four times its volume of ice-cold acetone. The mixture was stirred for 30 min. and then centrifuged to remove an inactive precipitate. The acetone was removed from the clear supernatant liquid by distillation under reduced pressure and the resultant aqueous solution was concentrated to about one-tenth of its original volume by freeze-drying. This was the concentrate used for chromatographic analysis.

Paper chromatography. One or more spots of the concentrates and one spot of a marker solution, containing 1 µg. each of thiamine, thiamine monophosphate and thiamine pyrophosphate/ml., were applied to What-

man no. 1 filter paper. The vitamin B₁-active compounds present were separated by descending chromatography with 2 g. *p*-toluenesulphonic acid - 60 ml. *tert*-pentanol - 30 ml. water as the developing solvent [10, 4]. The chromatograms were developed for 48 hr. and then dried. The zones of vitamin B₁ activity were located either by spraying the paper with bismuth potassium iodide as described by Bartley [4] or by the bioautograph technique described below.

Microbiological method

Measurement of vitamin B₁ activity in whole milk and milk ultrafiltrates. The vitamin B₁ activity was extracted by heating 2 ml. of milk with 50 ml. N/30-H₂SO₄ at 100° for 30 min. After cooling, the pH was adjusted to 4.6, the total volume made to 100 ml. and the extract filtered. The filtrate was diluted (if necessary) to contain about 0.01 µg. thiamine/ml. and added to the assay tubes in duplicate at levels of 0.5, 1.0, 2.0 and 4.0 ml. The ultrafiltrates were diluted with water and added to the assay tubes without further treatment.

A stock solution of thiamine hydrochloride, containing 1 mg./ml., was diluted in M/30-phosphate - citrate buffer, pH 4.6, to a concentration of 0.01 µg./ml. The presence of buffer was found to prevent losses of thiamine by adsorption onto the glassware [6]. This standard thiamine solution was added to the assay tubes at levels of 0.5, 1.0, 2.0, 3.0 and 4.0 ml.

The volumes in all the tubes were made up to 5 ml. with distilled water, and 5 ml. double-strength basal medium, prepared as described by Bánhidi [3], were added. The baskets of tubes were sterilized by heating at 100° for 30 min., cooled and inoculated with *Lactobacillus fermenti* 36 (ATCC 9338). The inoculum was prepared by growing *Lb. fermenti* at 37° for 24 hr. in enriched culture medium [2]. The culture was centrifuged, resuspended in 10 ml. 0.9% (w/v) sterile saline, diluted a further ten-fold in sterile saline and one drop of this suspension added to each assay tube. After incubation for 18 hr. at 37°, growth in the tubes was measured turbidimetrically and the vitamin B₁ activities of the test extracts were obtained by reference to the standard response curve.

Bioautographs. 150 ml. of basal assay medium [3] at single strength in 2% agar, were sterilized in a 250 ml. conical flask by heating at 110° for 10 min. The flask was transferred from the autoclave to a water bath held at 48° and allowed to stand for 45 min. to cool to the temperature of the bath. 50 mg. triphenyltetrazolium chloride, dissolved in 4 ml. sterile distilled water, were added to the medium so that the growth zones would show up red on a colourless background [7]. 3 ml. of a cell suspension of *Lb. fermenti* were then added. This suspension was

prepared by centrifuging a 24 hr. culture grown at 37° in 10 ml. of the basal assay medium supplemented with 0.05 µg. thiamine hydrochloride/ml. and then resuspending the cells in 10 ml. of 0.9% (w/v) saline. Immediately after these additions had been made the agar was poured into a covered sterile glass plate measuring 22 × 37 cm. When the plate and agar had cooled, the paper chromatogram was placed on the agar and the plate incubated at 37° for 18 hr. The red zones of growth indicated the positions on the chromatogram of compounds with vitamin B₁ activity for *Lb. fermenti*.

Measurement of cocarboxylase. Cocarboxylase (thiamine pyrophosphate) was measured manometrically by the method of Singer & Pensky [16], which is based on the decarboxylation of pyruvate by an α -carboxylase from wheat germ. The α -carboxylase was prepared from a sample of low-fat, unheated wheat germ kindly given to us by Joseph Rank Ltd. For this method of estimation it was necessary to add to the Warburg flasks 0.5 ml. of test solution containing about 0.1 µg. cocarboxylase.

RESULTS

Measurement of total vitamin B₁ activity in milks of different species

The assay organism, *Lb. fermenti*, can utilize for growth thiamine, its monophosphate or its pyrophosphate. Under the conditions of assay described here, all three compounds were equally active on an equimolar basis. Thus, values for total vitamin B₁ activity were obtained for the different milks and are shown in Table 1. The vitamin B₁ activities of the ultrafiltrates prepared from these milks are also given in the table. These ultrafiltrates were those used to prepare the concentrates for chromatographic analysis. In general, they contained 70% or more of the total activity present in the whole milk.

Chromatographic studies

Separation of thiamine, thiamine monophosphate and thiamine pyrophosphate. Fig. 2 shows a diagrammatic representation of the separation of a mixture of pure thiamine, thiamine monophosphate and thiamine pyrophosphate by chromatography on paper with the solvent system described in the experimental section. It was found necessary that at least 10 µg. of each thiamine compound be present on the paper to show it as a coloured spot after spraying with the bismuth potassium iodide reagent. This quantity would be contained in about 20 ml. of raw whole milk. Consequently, the method was quite unsuitable for detection of the vitamin B₁ active compounds in milk extracts. Fortunately the test could be made more sensitive by using the bioautograph technique,

Table 1

Vitamin B₁ activity of whole milks and milk ultrafiltrates of different species

The results are expressed as $\mu\text{g.}$ thiamine hydrochloride/ml. Test organism *Lactobacillus fermenti*.

Type of milk	Whole milk	Milk ultrafiltrate
Cow's		
Early lactation (2nd week)	0.83	0.77
Mid lactation (21st week)	0.44	0.32
Late lactation (39th week)	0.29	0.24
Human		
Early lactation	0.13	0.10
Sow's		
Mid lactation	0.73	0.52
Goat's		
Mid lactation	0.47	0.49
Rhinoceros's		
Late lactation	0.68	0.72

when as little as $0.1 \mu\text{g.}$ of each of the compounds could be detected. The chromatographic analysis of the "pure" compounds showed (Fig. 2.) that the sample of thiamine monophosphate contained a small amount

THIAMINE
HYDRO- MONO- PYRO-
CHLORIDE PHOSPHATE PHOSPHATE

Fig. 2. Separation of thiamine, thiamine monophosphate and thiamine pyrophosphate by chromatography in *p*-toluenesulphonic acid - *tert.*-pentanol - water. This and the following figures are tracings taken from the bioautograph plates. The experimental conditions are described in the text.



of free thiamine, and that the sample of thiamine pyrophosphate contained some monophosphate. The R_F values were: thiamine 0.61, thiamine monophosphate 0.41, and thiamine pyrophosphate 0.20.

Identification of vitamin B₁ active compounds present in milk concentrates. A concentrate, prepared from the sample of milk collected from a cow in the second week of lactation, was analysed for vitamin B₁

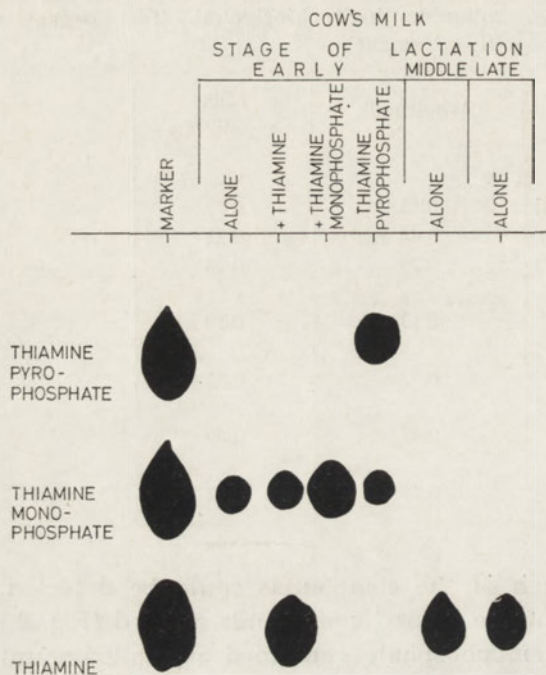


Fig. 3. Separation and identification of the vitamin B₁-active compounds present in cow's milk from early, mid and late lactation.

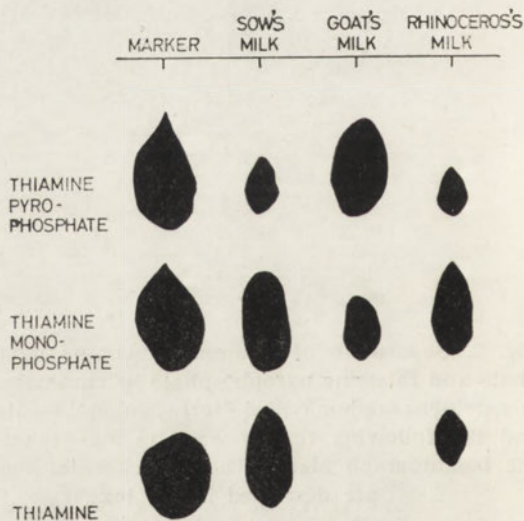


Fig. 4. Separation and identification of the vitamin B₁-active compounds present in the milk of the sow, goat and rhinoceros.

active compounds by the paper chromatography and bioautograph techniques. The bioautograph is shown in Fig. 3. Thiamine monophosphate was the only compound found to be present. The addition of thiamine,

thiamine monophosphate or thiamine pyrophosphate to the concentrate confirmed that the spot was due to the monophosphate and was not an artifact caused by some component present in the concentrate slowing up the movement of free thiamine. The concentrates prepared from mid- or late-lactation cow's milk were found to contain only free thiamine.

Similar analyses of concentrates prepared from the milk of the sow, goat and rhinoceros showed interesting differences between the milks of these different species (Fig. 4). In sow's milk (from mid lactation), about equal amounts of thiamine and thiamine monophosphate were present together with some thiamine pyrophosphate. The goat's milk sample (also from mid lactation) contained more of the pyrophosphate than of the monophosphate and no free thiamine. In the concentrate of rhinoceros's milk all three compounds were present. There was not enough vitamin B₁ activity in the human milk sample for any to be detected in the concentrate.

Coccarboxylase estimations

No coccarboxylase activity could be detected in the ultrafiltrates prepared from the early, mid- or late-lactation cow's milk samples. A quantitative recovery of coccarboxylase added to the milk ultrafiltrates showed that their lack of activity was not due to the presence of inhibitory substances such as inorganic pyrophosphate [see 17].

The milks of the sow, goat, woman and rhinoceros were analysed at a later date and regrettably no manometric coccarboxylase estimations were done.

DISCUSSION

The low total vitamin B₁ activity of milk makes the separation and identification of the individual thiamine compounds a difficult problem. Several methods, some involving ion-exchange resins, have been proposed for separating thiamine and its phosphates [15, 8, 13, 14] but, although these methods have worked well with the pure compounds or with animal tissues rich in vitamin B₁ activity, in our experience none of them proved satisfactory for milk. The simple technique of ultrafiltration, treatment with acetone and concentration by freeze-drying yielded a milk concentrate with high vitamin B₁ activity and containing no substances that interfere with the paper chromatographic analysis. Admittedly the extraction of the vitamin was not quantitative. In the first place, any vitamin B₁ active compounds firmly bound to protein would not be present in the ultrafiltrate. According to Houston *et al.* [11] some 15% of the total vitamin B₁ activity of cow's milk is protein-bound. However, the main purpose of our experiments was to identify the phosphorylated form of thiamine present in cow's milk,

which we were able to do. Our results support de Jong's [12] supposition that it is the monophosphate and not the pyrophosphate (cocarboxylase) that is present.

The presence of considerable quantities of both the monophosphate and pyrophosphate in the milk of the goat and pig may be attributed to the lower phosphatase contents of these milks [11, 5]. Previous reports from this Institute have shown that only about 10% of the total vitamin B₁ activity of sow's and goat's milks was free thiamine [11, 5]. De Jong [12] also reported low levels of free thiamine in goat's milk, but he found no cocarboxylase and therefore assumed that, as in cow's milk, the phosphorylated vitamin form was thiamine monophosphate. The fact that we could detect both the monophosphate and the pyrophosphate in goat's milk and the fact that de Jong found that cocarboxylase added to his sample of goat's milk was converted to the monophosphate suggests that his sample might have been taken at the end of the lactation when the phosphatase level increases, or that it was contaminated with bacteria having high phosphatase activity.

The high proportion of the mono- and pyro-phosphates of thiamine present in the sow's and goat's milks, known to be low in phosphatase activity, lends support to the suggestion that vitamin B₁ is secreted in milk as cocarboxylase and is subsequently dephosphorylated by the milk enzymes. The proportions of the different forms of the vitamin present would then depend on the amounts and types of phosphatases present in the milk, and would vary both from species to species and with the stage of lactation.

SUMMARY

A method is described for identifying the vitamin B₁-active compounds present in milk. Thiamine monophosphate was found to be present in early-lactation cow's milk, whereas only free thiamine was present in mid- or late-lactation milk. Both free thiamine and its mono- and pyrophosphates were present in the bulk sample of mid-lactation sow's milk and in a sample of rhinoceros's milk taken at the end of lactation. Mid-lactation goat's milk contained a high proportion of thiamine pyrophosphate, some thiamine monophosphate but no free thiamine. The possible relationship between the proportion of phosphorylated thiamine present and the phosphatase activity of the milks is discussed.

REFERENCES

- [1] Aschaffenburg R., Gregory M. E., Rowland S. J., Thompson S. Y. & Kon V. M. - *Proc. zool. Soc. London* **137**, 475, 1961.
- [2] Association of Vitamin Chemists, Inc., *Methods of Vitamin Assay*, 2nd ed Interscience Publishers, Inc., New York 1951.

- [3] Bánhidi Z. G. - *Acta chem. Scand.* **12**, 517, 1958.
- [4] Bartley W. - *Biochem. J.* **56**, 379, 1954.
- [5] Braude R., Coates M. E., Henry K. M., Kon S. K., Rowland S. J., Thompson S. Y. & Walker D. M. - *Brit. J. Nutr.* **1**, 64, 1947.
- [6] Farrer K. T. H. & Hollenberg W. C. J. - *Analyst* **78**, 730, 1953.
- [7] Ford J. E. & Holdsworth E. S. - *Biochem. J.* **53**, XXII, 1953.
- [8] de Giuseppe L. & Rindi G. - *J. Chromat.* **1**, 545, 1958.
- [9] Gregory M. E. - *Brit. J. Nutr.* **8**, 340, 1954.
- [10] Hanes C. S. & Isherwood F. A. - *Nature, Lond.* **164**, 1107, 1949.
- [11] Houston J., Kon S. K. & Thompson S. Y. - *J. Dairy Res.* **11**, 151, 1940.
- [12] de Jong S. - *Enzymologia* **10**, 253, 1942.
- [13] Rindi G. & de Giuseppe L. - *Biochem. J.* **78**, 602, 1961.
- [14] Rossi-Fanelli A., Ipata P. L. & Fasella P. - *Biochem. Biophys. Res. Comm.* **4**, 23, 1961.
- [15] Siliprandi D. & Siliprandi N. - *Biochim. Biophys. Acta* **14**, 52, 1954.
- [16] Singer T. P. & Pensky J. - *J. Biol. Chem.* **196**, 375, 1952.
- [17] Wiethoff E. O., Leppla W. & Holt C. V. - *Biochem. Z.* **328**, 576, 1957.

FORMY UFOSFORYLOWANEJ TIAMINY W MLEKU RÓŻNYCH ZWIERZĄT

Streszczenie

Opisano metodę identyfikacji znajdujących się w mleku związków czynnych jako witamina B₁. W mleku krowy na początku laktacji stwierdzono obecność monofosforanu tiaminy, natomiast w środkowym okresie i przy końcu laktacji jedynie obecność wolnej tiaminy. W mleku świni w środkowym okresie laktacji i w mleku nosorożca pobranym przy końcu laktacji znaleziono zarówno wolną tiaminę, jak i mono- i pyrofosfotiaminę. Mleko kozy w środkowym okresie laktacji zawierało znaczną ilość pyrofosfotiaminy i trochę monofosfotiaminy, natomiast nie stwierdzono w nim wolnej tiaminy. Przedyskutowano przypuszczalną zależność między zawartością ufosforylowanej tiaminy a aktywnością fosfatazy w mleku.

Received 9 December 1963.

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THERMAL REACTIVATION OF ULTRAVIOLET INACTIVATED BACTERIOPHAGES

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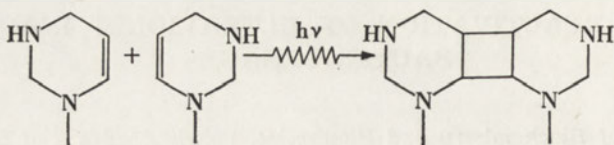
The phenomenon of biological photoreactivation (PR)¹ is fairly widespread in nature and has been the subject of numerous investigations (for recent reviews see [10, 19, 17, 20]). Photoreactivation amongst the bacteriophages was first reported by Dulbecco [5], who observed that ultraviolet irradiated phage, following adsorption to their host cells, could be partially reactivated by exposure to visible light. It is now reasonably well established that most instances of photoreactivation represent a direct reversal by visible light of some modifications ("lesions") produced in essential nucleic acid molecules by the action of ultraviolet radiation. Consequently, aside from its biological significance, the phenomenon provides a supplementary tool for detailed investigations of the nature of the lesions induced by UV light. The potential significance of PR is further emphasized by the demonstration that it is the result, at least in large part, of some light-activated enzymic reaction (for review see [17]).

Considerably less interest has been devoted to the fact that ultraviolet induced damage may also frequently undergo partial reversal in the dark by an increase in temperature at neutral pH, a finding first reported by Anderson [2] and Stein & Meutzner [21] for *E. coli*, and subsequently by Bresch [4] for coliphage T₁ adsorbed to its host cell.

Attention has previously been drawn to the significance of TR in the bacteriophages and the importance of confirming the finding of Bresch [4] for T₁ phage, as well as examining whether it applies to other phages and, in particular, the T-even bacteriophages [19]. Physico-chemical investigations of the nature of the radiation-induced damage in natural nucleic acids and model oligo- and polynucleotides have demonstrated that practically all of this "damage" is due to modifications

¹ PR and TR will be used in this text for photoreactivation and thermal reactivation, respectively.

of the pyrimidine rings, for radiation doses in the range of those responsible for biological effects. One of the known reactions involves saturation of the 5,6 double bonds of uracil and cytosine residues with concomitant uptake of a water molecule to form the 5-hydro-6-hydroxy derivatives; this reaction is heat reversible in the dark at neutral pH. Another reaction may take place between adjacent uracil and/or thymine residues, with the formation of a cyclobutane ring between the 5,6 bonds, as follows:



This reaction is not reversible by heat but may be reversed by UV both for the free dimers [3] as well as in polynucleotide chains [12, 11]. Furthermore it has been demonstrated that PR in isolated nucleic acids is due in large part to a light-activated enzymic reversal of this dimerization reaction between thymine residues [17, 20].

The T-even bacteriophages fall into a special category in that, in place of cytosine, they contain only 5-hydroxymethylcytosine and glucosylated 5-hydroxymethylcytosine. These derivatives do not form heat reversible photoproducts in the free state, although there is some evidence indicating that their photoproducts are potentially capable of reverting to the parent compounds in the dark [25].

It becomes of interest, therefore, to examine the extent to which various ultraviolet inactivated bacteriophages may be thermally reactivated in the dark, whether adsorption to the host cells is indeed a prerequisite for such reactivation and, particularly, what, if any, is the relationship of TR to PR. Such observations would be expected to be of value in any direct comparison of the biological and physico-chemical effects resulting from ultraviolet irradiation.

MATERIALS AND METHODS

Most of the experiments were carried out with phages T₁ and T₂, using *E. coli* B as host cell. The synthetic medium of Schulmeister (Na₂HPO₄ · 12H₂O, 11.48 g.; KH₂PO₄, 1.08 g.; (NH₄)₂SO₄, 0.8 g.; MgSO₄ · 7H₂O, 0.08 g.; CaCl₂, 0.002 g.; FeSO₄ · 7H₂O, 0.001 g. per liter of glass distilled water, pH 7.4) supplemented with 0.5% glucose, was employed throughout. Several orientative experiments were also carried out with other phages as well as with *E. coli* K 12 as host cell.

Phage were grown on *E. coli* B in liquid, synthetic medium as described by Adams [1] at a multiplicity of infection of 0.3. The phage

preparations were stored under chloroform at 4°. Phage titrations were carried out by the dilution method according to Takatsy [23] with calibrated spiral loops. The same procedure was used to determine the number of infective centres.

E. coli B cells from an agar slant were used to inoculate a synthetic medium which was incubated overnight at 37° in stationary culture. About 4 ml. of this cell suspension was transferred to 50 ml. fresh medium and this was incubated on a shaker to an optical density of 0.15 at a wavelength of 650 mμ, corresponding to about 2.4×10^8 cells/ml. This suspension was employed for phage adsorption and reactivation.

A Phillips 6-watt bactericidal lamp was employed for phage inactivation. The phage suspension, suitably diluted to 10 ml. in synthetic medium, was contained in a 9.5 cm. Petri dish at a layer thickness of 0.1 cm. and was stirred continuously during irradiation. Except where otherwise indicated, inactivation was carried out to about 99.7%. This required 50 sec. irradiation for phage T₂ at a distance of 20 cm. from the lamp; for phage T₁ the corresponding time was 150 sec.

For thermal reactivation a suitable volume of the above-mentioned *E. coli* cell suspension was added to an aliquot of the irradiated phage suspension so as to obtain the desired multiplicity of infection. Following mixing the suspension of adsorbed phage was divided into two portions, one of which was immersed in a bath at 37° and other at 45°. At given time intervals, 1 ml. samples were removed from each flask and immediately diluted with synthetic medium. Following addition of detector and liquid agar, the samples were poured into plastic dishes, incubated at 37° and 18 hr. for phage T₂, and 8 hr. for T₁, and the number of plaques counted.

All experiments were conducted under a red lamp in the dark room in order to avoid interference from photoreactivation.

RESULTS

Thermal reactivation of phage T₂. A phage suspension containing 9.4×10^9 infective particles per ml. was diluted 1000-fold with synthetic medium and inactivated by irradiation to a residual activity of 0.3%. The inactivated phage was then adsorbed to a suspension of young cells at a multiplicity of infection of 3, and the resulting suspension divided into two portions which were incubated at 37° and 45°, respectively. Non-irradiated controls were incubated simultaneously at both temperatures. At intervals of 5, 10, 30 and 45 min. samples were collected from all four flasks for estimation of viable phage particles.

From the results shown in Fig. 1, it is clear that the survival of irradiated phage is much more marked at 45° than at 37°. It should also be noted that the number of surviving phage particles at 45° varies with

time of incubation, increasing to a maximum during the first 10 min. and then decreasing so that after 45 min. the number of infectious particles is again equal to that at zero time. On the other hand, at an incubation temperature of 37° the titer of active phage increases much less,

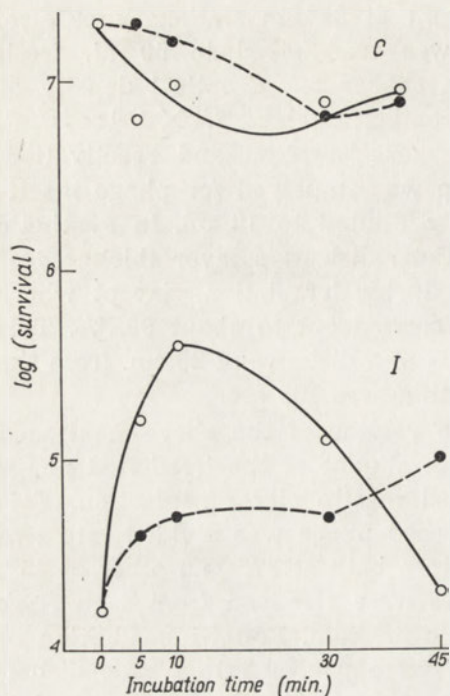


Fig. 1. Thermoreactivation of ultraviolet-inactivated phage T_2 : C, non-irradiated controls; I, UV-inactivated; (○), incubation at 45°; (●), incubation at 37°.

exhibiting only slight reactivation with no subsequent inactivation with time of incubation. For the non-irradiated control, incubation at 45° results in a slow, but continual, inactivation with time.

Account must here be taken, of course, of the known fact that T_2 normally exhibits marked multiplicity reactivation [13]. It is consequently somewhat surprising that a freshly prepared phage stock shows only a minimal degree of multiplicity reactivation by comparison with a phage stock which has been stored for some time prior to use, while the degree of TR is the same for both (see Fig. 3).

It was observed during the above experiments that the thermal reactivation of irradiated phage is more effective if the host cells are taken from an actively growing culture. There is, on the other hand, no difference in the course or degree of thermoreactivation if the adsorption and reactivation of phage are carried out on a glucose-free medium. It would therefore seem that the temperature reactivation of irradiated phage is linked in some way with the metabolic state of the host cells at the time of thermoreactivation. But the ability of the host cells to

induce reactivation is dependent on their metabolic state prior to adsorption of the phage; modifications following infection apparently are of little importance.

The course of TR with time of incubation for phage T_2 is exhibited in Fig. 2. Reactivation at 45° is a relatively rapid process, attaining an apparent maximum at about 6 min. and remaining at this level for about 25 min., following which inactivation sets in. At 37° there is only slight reactivation for up to 20 min., followed by an increase which is undoubtedly due to normal phage synthesis. At 45° phage synthesis is inhibited.

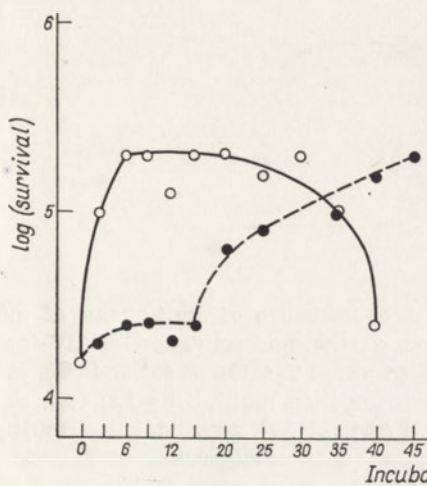


Fig. 2

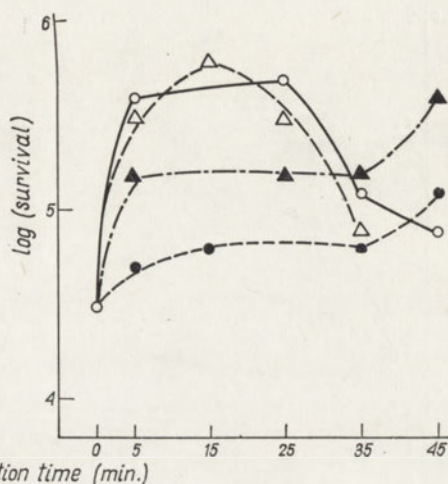


Fig. 3

Fig. 2. Course of thermoreactivation of ultraviolet-inactivated phage T_2 with time, (O), at 45° and (●), at 37° .

Fig. 3. Course of thermoreactivation of fresh and stored (hence partially spontaneously inactivated) UV-inactivated phage T_2 preparations: (O), at 45° and (●), at 37° for fresh (2-day-old) preparation; (Δ), at 45° and (\blacktriangle), at 37° for older preparation.

In all probability the degree of TR is greater than that observed in Fig. 1 at 45° , hence the use of the term "apparent maximum" in the previous paragraph. What is most likely being observed is the difference between phage reactivation and heat inactivation.

It was also noted that the course of reactivation of irradiated phage is dependent, to a considerable extent, on the age of the bacteriophage preparation. This is demonstrated in Fig. 3, which exhibits the course of TR for two phage preparations, one two days old, the other seven weeks, conducted under identical conditions. It will be observed that the degree of TR at 45° is qualitatively similar for both phage prepa-

rations. There is, by contrast, a marked difference at 37°, where the older preparation exhibits more pronounced multiplicity reactivation.

Role of coefficient of infection on degree of thermoreactivation. The foregoing experiments were carried out at a multiplicity of infection of 3 so that the majority of the host cells were multiply infected. It is consequently conceivable that some type of multiplicity reactivation may be responsible for TR. The degree of TR was therefore examined at various multiplicities of infection, and it was in fact found that the higher the multiplicity of infection, the greater the degree of TR. This is illustrated in Fig. 4 which exhibits the degree of TR at multiplicities

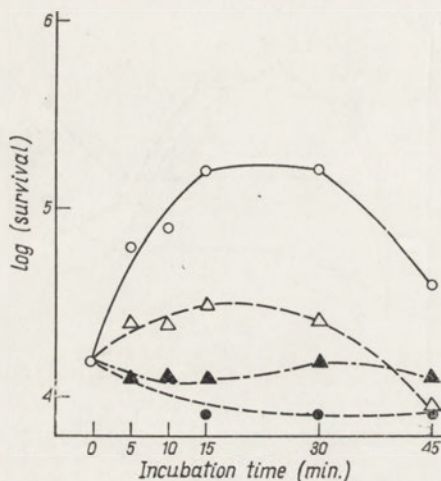


Fig. 4. Influence of multiplicity of infection on thermoreactivation of UV-inactivated phage T₂: (○), at 45° and (●), at 37° with infection multiplicity 2.2; (Δ), at 45° and (▲), at 37° with infection multiplicity 0.044.

of infection of 0.044 and 2.2, and demonstrates conclusively that there is practically no observable TR under conditions where most host cells are singly infected. It must therefore be concluded that the observed TR is not due to the heat reactivation of individual UV-irradiated phage particles, i.e. TR is not the result of a direct reversal of UV-induced lesions.

The dependence of TR on the multiplicity of infection is also demonstrated indirectly by the difference in degree of thermoreactivation between fresh and old phage preparations at a multiplicity of infection of 0.06 (see Fig. 5). From Fig. 5A it will be seen that a 7-weeks old phage sample gives pronounced TR at an infection multiplicity of 0.06; whereas a fresh preparation exhibits practically no TR at the same infection multiplicity (Fig. 5B). By contrast, both samples exhibit the same degree of TR at an infection multiplicity of 2.9. Since, in the older preparation, the titer of active phage particles was significantly lowered, the pronounced degree of TR observed at an infection multiplicity of 0.06 can be ascribed to the presence of inactive phage particles which participate in thermoreactivation, the actual "infection multipli-

city" being then higher than that calculated from the number of active phage particles in the preparation.

Attempted thermoreactivation of phage T_1 . In view of the fact that the degree of TR for irradiated T_2 is a function of the coefficient of infection, suggesting some relationship to the phenomenon of multiplicity reactivation, TR was sought for in irradiated phage T_1 which does not exhibit multiplicity reactivation [13].

Experiments were carried out in a manner completely analogous to those for T_2 . In different experiments TR was attempted with coefficients of infection of 0.04 and 1.6 as well as 0.04 and 3.0. No evidence for TR

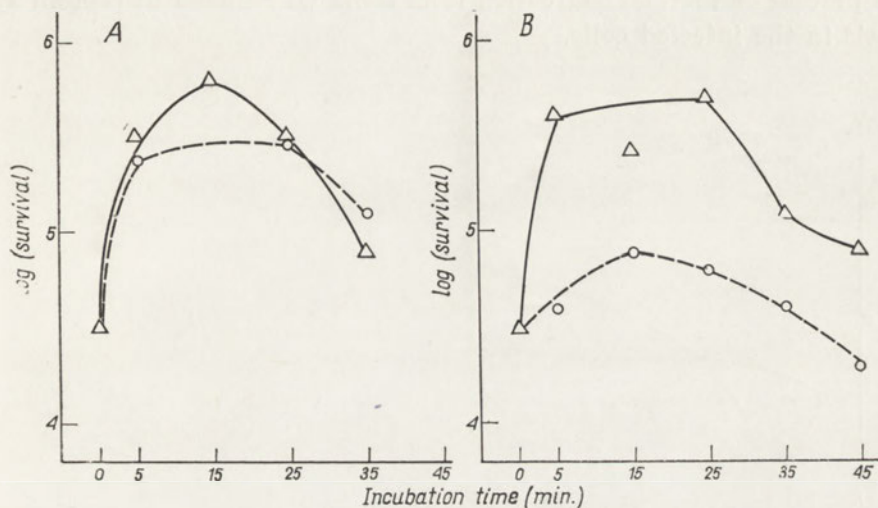


Fig. 5. Course of thermoreactivation for UV-inactivated T_2 as a function of age of phage sample and multiplicity of infection; at 45° : A, phage sample 7 weeks old; B, fresh phage sample. (O), infection multiplicity 0.06; (Δ), infection multiplicity 1.6.

could be found at coefficients of infection greater than 1 at 45° ; at coefficients of infection of 0.01 to 0.04 the slight reactivation observed (Fig. 6) was considered too small to be of significance, although it was of the same order of magnitude as that reported by Bresch [4] for thermoreactivation at 45° using a multiplicity of infection of 0.12 to 0.2. This may be construed as additional evidence in support of the contention that TR for phage T_2 is due to some form of multiplicity reactivation.

Influence on TR of phage T_2 of inhibitors of protein and DNA synthesis. It is well established that protein synthesis plays an important role during the process of phage multiplication [6, 14, 24, 8], and phage synthesis is inhibited if an inhibitor of protein synthesis is present at the moment of infection. It was consequently of interest to examine whether TR of irradiated phage is linked with protein synthesis.

Chloromycetin was used as an inhibitor of protein synthesis, at a final concentration of 50 $\mu\text{g./ml.}$, and added to the medium at the moment of infection as well as 5, 15 and 30 min. prior to infection, at a temperature of 37° . It was found that if chloromycetin was added at the moment of infection, TR was very similar to that of a control. Incubation of the host cells with chloromycetin prior to infection resulted in a decrease in TR, the extent of which was dependent on the time of incubation of the host cells with chloromycetin prior to infection. For the periods of incubation employed, i.e. 5, 15 and 30 min., the per cent inhibitions of TR were 60%, 88%, and 92% respectively. It therefore follows that the process of thermoreactivation is in some way linked to protein synthesis in the infected cells.

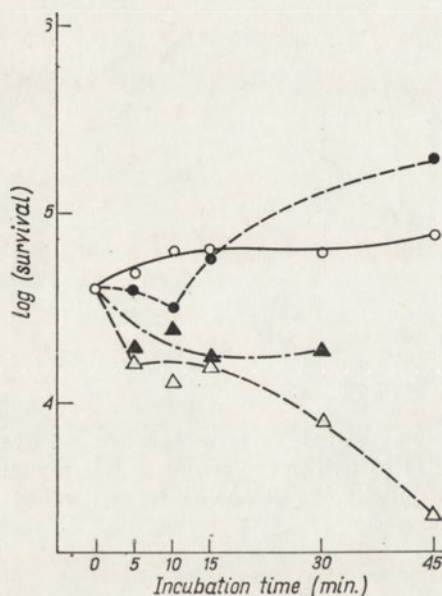


Fig. 6. Attempted thermoreactivation for UV-inactivated phage T_1 : (O), at 45° and (●), at 37° with infection multiplicity 0.01; (Δ), at 45° and (▲), at 37° with infection multiplicity 1.6.

Mitomycin C [18] and 5-fluorodeoxyuridine [16] were used as inhibitors of DNA synthesis, at final concentrations of 2 $\mu\text{g./ml.}$ and 10 $\mu\text{g./ml.}$, respectively. The host cells were incubated with the inhibitors at 37° for 30 min. prior to infection, controls being kept during this period in the cold room. Subsequently, following adsorption of control and irradiated phage, TR was carried out at a multiplicity of infection of 4.3. It was found that mitomycin C, although greatly reducing the colony-forming ability of the host cells, gave no inhibition of TR. On the other hand fluorodeoxyuridine, which affects the colony-forming ability of the host cells to a much smaller degree, inhibited TR to the extent of about 60%.

DISCUSSION

Of the various types of restoration of UV damage hitherto reported in microorganisms, that which bears a fairly close resemblance to photo-reactivation (PR) is thermoreactivation (TR) [2]. However, it has now been reasonably well established that PR is due, at least in large part, to the light-induced enzymic reversal of UV lesions (presumably thymine dimers) in the case of transforming DNA [17]. There is also some indirect evidence that PR for bacteriophages follows the same pathway [7]. On the other hand nothing concrete is as yet known regarding the nature of the mechanism of thermoreactivation, although there are some indications that for UV-inactivated *E. coli* B and *S. cerevisiae* TR reverses the same damage as PR [9].

From the results obtained in the present investigation it would appear that TR for UV-inactivated bacteriophages bears little, if any relationship to PR and that is is, in effect, more complex. It would, of course, be desirable to obtain quantitative data for the extent of PR prevailing following increasing degrees of UV inactivation. Such experiments are in progress and preliminary results indicate that the course of TR differs in some respects from that for PR. Furthermore, while the absolute magnitude of the TR effect in T_2 is somewhat less than that for PR, it is necessary to emphasize once more that the experimentally observed TR probably represents only a minimum value. The fact that no TR can be placed in evidence with singly infected cells, using freshly prepared phage, provides perhaps the strongest evidence for a different mechanism for TR as compared to PR.

Bearing in mind that phage T_1 , which is known to exhibit multiplicity reactivation only to a minimal degree [13], likewise exhibits only minimal TR as compared to phage T_2 , it seems not unreasonable to infer that the TR observed for T_2 and several other phages is due to some form of temperature induced multiplicity reactivation. Such an inference is supported by the difference in the degree of TR between fresh and old phage preparations at an infection multiplicity of 0.06 and the absence of any difference at an infection multiplicity of 2.9. This follows since the titer of active phage particles decreases during storage so that the multiplicity of infection, calculated on the basis of the total number of phage particles, is increased.

The kinetics of TR for phage T_2 at 45° suggest that reactivation proceeds during the phase preceding phage synthesis. It may therefore be inferred that the reactivation process is not the result of genetic recombination during replication of phage DNA, as proposed by Luria [13] for multiplicity reactivation. Meselson & Weigle [15] have in fact proposed, in the case of the phage λ , that at elevated multiplicities of

infection one may encounter recombination without separation of the DNA strands and without replication.

However, considerably more experimental data will be required to delineate the nature of the mechanism involved in thermoreactivation. It should be noted in this connection that a variety of different mechanisms have already been reported to effect repair of ultraviolet damage in bacteriophages, all of them requiring an intracellular environment [7]. The TR reported here for phage T_2 has nothing in common with the TR reported by Kaplan *et al.* [12] for reversal of c-mutations in phage K of *Serratia marcescens*. The latter process was found to be effective on extracellular phage and was ascribed to thermal reversal of cytosine hydration (see ref. [19]). All our attempts to demonstrate TR in extracellular T_2 were completely negative.

Preliminary experiments have demonstrated that T_1 and T_3 do not exhibit TR, while T_4 undergoes TR to about the same extent as T_2 . Furthermore TR for phage T_4 appears to be even greater in magnitude than PR. These findings are the object of more detailed studies. It should also be mentioned in this connection that Bresch (personal communication) has found evidence for TR in phage T_7 .

SUMMARY

Ultraviolet inactivated bacteriophage T_2 may be partially reactivated thermally at a temp. of 45° following adsorption to the host cell *E. coli* B. Other T phages also exhibit some thermoreactivation, but to a lesser degree than T_2 . Attempts to obtain thermoreactivation outside the cellular environment were negative.

Thermoreactivation for phage T_2 was most pronounced following prior UV inactivation to a residual activity of 1% or less. The degree of thermoreactivation attainable, an order of magnitude or greater, was somewhat less than for photoreactivation. Pronounced thermoreactivation was observed only in multiply infected cells. The overall findings indicate that the thermoreactivation observed bears little, if any, relationship to photoreactivation, but is rather some form of temperature induced multiplicity reactivation.

The influence, on the thermal reactivation process, of inhibitors of protein and nucleic acid syntheses, was also examined; and the results are discussed in relation to photoreactivation and other mechanisms for reversal of ultraviolet injury in bacteriophages.

REFERENCES

- [1] Adams M. H., *Bacteriophages*, Interscience Publishers Inc. New York, 1959.
- [2] Anderson E. H. - *J. Bacteriol.* **61**, 389, 1950.
- [3] Beukers R. & Berends W. - *Biochim. Biophys. Acta* **49**, 181, 1960.

- [4] Bresch C. - *Z. Naturforsch.* **5b**, 420, 1950.
- [5] Dulbecco R. - *J. Bacteriol.* **59**, 329, 1950.
- [6] Edlinger E. - *Ann. Inst. Pasteur* **81**, 514, 1951.
- [7] Harm W., in *Repair from Genetic Radiation*, Pergamon Press, Oxford, 1963.
- [8] Hershey A. D. & Melechen N. E. - *Virology* **3**, 207, 1957.
- [9] Jagger J. - *Bacteriol. Rev.* **22**, 99, 1958.
- [10] Jagger J., in *Radiation Protection and Recovery* (A. Hollaender, ed.) Pergamon Press, London, 1960.
- [11] Johns H. E., Rapaport S. A. & Delbruck M. - *J. Mol. Biol.* **4**, 104, 1962.
- [12] Kaplan R. W., Winkler U. & Wolff-Ellmänder H. - *Nature*, **186**, 330, 1960.
- [13] Luria S. E. - *Proc. Natl. Acad. Sci. U.S.* **33**, 253, 1947.
- [14] Melechen N. E. - *Genetics* **40**, 585, 1955.
- [15] Meselson M. & Weigle J. J. - *Proc. Natl. Acad. Sci. U.S.* **47**, 857, 1961.
- [16] Rich M. A., Bolaffi J. L., Knoll J. E., Cheong L. & Eidinoff M. L. - *Cancer Res.* **18**, 730, 1958.
- [17] Rupert C., in *Photobiology, Action of Light on Living Materials* (A. C. Giese, ed.) Academic Press, New York, 1963.
- [18] Shiba S., Terawaki A., Taguchi T. & Kawameta J. - *Nature* **183**, 1056, 1959.
- [19] Shugar D., in *The Nucleic Acids* (E. Chargaff & J. N. Davidson, eds.) Academic Press, Inc., New York, 1960.
- [20] Smith K. C., in *Photobiology, Action of Light on Living Materials* (A. C. Giese, ed.) Academic Press, New York, 1963.
- [21] Stein W. & Meutznier J. - *Naturwissen.* **37**, 167, 1950.
- [22] Sztumpf E. & Shugar D. - *Biochim. Biophys. Acta* **61**, 555, 1962.
- [23] Takátsy G. - *Kisérletes Orvostudomány* **4**, 60, 1952.
- [24] Tomizawa J. & Sunakawa S. - *J. Gen. Physiol.* **39**, 553, 1956.
- [25] Wierzchowski K. & Shugar D. - *Acta Biochim. Polon.* **7**, 63, 1960.

REAKTYWACJA TERMICZNA BAKTERIOFAGÓW INAKTYWOWANYCH PROMIENIAMI UV

Streszczenie

Bakteriofag T₂ inaktywowany działaniem promieni UV może być reaktywowany w temp. 45° jedynie po uprzedniej jego adsorpcji do komórki gospodarza (termoreaktywacja). Pozostałe fagi serii T są także zdolne do termoreaktywacji, ale w znacznie mniejszym stopniu niż fag T₂. Zjawisko termoreaktywacji faga T₂ w znacznym stopniu występuje tylko wtedy gdy fag był uprzednio inaktywowany w co najmniej 99%. Termoreaktywacja zachodzi tylko w komórce zakażonej więcej niż jednym fagiem. Stopień reaktywacji inaktywowanego faga pod wpływem temp. 45° jest nieznacznie niższy w porównaniu z fotoreaktywacją.

Badano wpływ inhibitorów syntezy białka i DNA na proces termoreaktywacji. Wyniki badań przeprowadzonych nad zjawiskiem termoreaktywacji dyskutowano w odniesieniu do zjawiska fotoreaktywacji i innych mechanizmów reperacji uszkodzeń wywołanych działaniem promieni UV w bakteriofagach.

Received 18 December 1963.

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HYDROXYLATION OF PHENYLALANINE IN INSECTS AND SOME VERTEBRATES

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The hydroxylation of phenylalanine to tyrosine has been demonstrated by Udenfriend & Cooper [16] in homogenates of rat liver in the presence of NADH_2 . The mechanism of this reaction in mammals was explained by Kaufman *et al.* [7 - 13] who demonstrated that the enzymic system isolated from rat liver consists of two protein fractions. Both of these fractions, as well as a natural cofactor present in the deproteinized extract from rat liver, and NADH_2 participate in the hydroxylation reaction. It was found that dihydropteridine is the natural cofactor [11]. Fraction I is the proper enzyme introducing the hydroxyl group to phenylalanine in the presence of tetrahydropteridine. The action of the second enzyme (fraction II) consists in reducing dihydropteridine with NAD(P)H_2 to tetrahydropteridine; sheep liver is the richest source of this enzyme.

The formation of tyrosine from phenylalanine in insects was first demonstrated in 1956 by Fukuda [5] and Bricteux-Grégoire *et al.* [3], who injected the caterpillars of the silkworm *Bombyx mori* with labelled phenylalanine and found almost the whole of the introduced radioactivity in the tyrosine of the silk protein. However, it is not clear whether the mechanism of the hydroxylation reaction in insects is the same as in mammals.

MATERIALS AND METHODS

Animals. Insects: *Celerio euphorbiae*, *Bombyx mori* and *Ephestia Kühniella* (Lepidoptera), *Calliphora* (Diptera), *Dixippus morosus* (Dermaptera). Vertebrates: carp, frog, pigeon and rat.

Assays on crude extracts. The whole insect or the liver of a vertebrate was homogenized with 0.9% KCl, and centrifuged for 60 min. at 0° at 18 000 *g*; the supernatant was dialysed at $0 - 3^\circ$ for 3 hr. under shaking against a constant flow of a solution of 0.9% KCl in 0.01 M-sodium - potassium phosphate buffer, pH 7.0. Hydroxylase activity in the

whole extract was determined according to Udenfriend & Cooper [16]. Conditions and composition of the incubation mixture are given in Table 1.

Purified enzyme preparations. Fraction I was purified according to Kaufman & Levenberg [13]. The whole insect or the liver of a vertebrate was homogenized in a Waring Blendor for 30 sec. with 1.5 vol. of 0.01 M-acetic acid and for further 30 sec. after adding the same volume of acetic acid. The obtained homogenate was centrifuged at 0° at 18 000 g for 45 min. The supernatant was fractionated with ethanol at -30°. The active fraction precipitated between 10 and 21% ethanol concentration was centrifuged, then dissolved in 0.033 M-phosphate buffer, pH 7.4, and $(\text{NH}_4)_2\text{SO}_4$ was added. The fraction precipitated on addition of 20.3 g. $(\text{NH}_4)_2\text{SO}_4$ per 100 ml. was discarded and to the supernatant $(\text{NH}_4)_2\text{SO}_4$ was added, 5.6 g. per 100 ml. After centrifugation, the sediment was dissolved in 0.033 M-phosphate buffer, pH 7.4, and dialysed against 50 vol. of the same buffer for 12 hr. at 4°.

Fraction II was obtained from sheep liver and purified according to Kaufman & Levenberg [13]. The liver was homogenized in a Waring Blendor with 1.5 vol. of 0.1 M-phosphate buffer, pH 7.4, for 1 min., and for a further minute after adding the same volume of the buffer. The homogenate was centrifuged for 45 min. at 18 000 g at 0°. To the supernatant twice 28 g. $(\text{NH}_4)_2\text{SO}_4$ per 100 ml. were added; the first sediment was discarded and the second one dissolved in 0.0167 M-phosphate buffer, pH 7.4, and dialysed for 12 hr. at 0° against 50 vol. of the same buffer. Further purification was carried out by ethanol and the fraction precipitated by 44-49% ethanol concn. at -8.5° was collected. The sediment was dissolved in 0.033 M phosphate buffer, pH 7.4, and dialysed against 50 vol. of 0.0167 M-phosphate buffer for 12 hr. at 0°.

All the steps of purification of both enzymic fractions were carried out at 2-4°. The purified preparations were stored at a temperature of -15° to -20°.

To obtain the "cofactor" of Kaufman [9] rat liver was homogenized in a Waring Blendor with 1.5 vol. of water, then deproteinized by pouring it slowly into 1.5 vol. of boiling water and boiling for a few minutes.

The enzymic activity of the purified preparations were determined according to Kaufman & Levenberg [13]. Conditions and composition of the incubation mixture are given in Table 2.

Analytical assays. Tyrosine formed during the reaction was determined by the colorimetric method according to Ceriotti & Spandrio [4]; protein was determined by the Mejbaum-Katzenellenbogen tannin method [14].

Special reagents. L-Phenylalanine, nicotinamide and folic acid (Biuro Obrotu Odczynnikami, Gliwice, Poland); L-tyrosine (Zakłady Farmaceutyczne, Warszawa); α -nitroso- β -naphthol and trichloroacetic acid (Fabry-

ka Odczynników Chemicznych, Gliwice); reduced diphosphopyridine dinucleotide (Nutr. Biochem. Corp., Cleveland, Ohio). Dihydrofolic acid was prepared according to Futterman [6]: 20 mg. of folic acid was suspended in 2 ml. of water and dissolved by adding 1 N-KOH. Next were added 5 ml. of 0.01% potassium ascorbate, pH 6.0, and 200 mg. $\text{Na}_2\text{S}_2\text{O}_4$. After 5 min., the solution was cooled to 0°, and brought to pH 2.8 with 2 N-HCl. The precipitated dihydrofolic acid was centrifuged, suspended in ascorbate solution and adjusted to pH 2.8. The sediment was washed 4 times with 10 ml. portions of cold 0.005 N-HCl. The obtained product dissolved in 0.1 N-KOH had the E_{283}/E_{340} m μ ratio of 2.8 - 3.0, characteristic for dihydrofolic acid. For experiments, the purified sediment of dihydrofolic acid was suspended in 5 ml. of 0.005 N-HCl, and 0.1 ml. samples were used.

RESULTS

The activity of phenylalanine 4-hydroxylase in crude extracts was assayed by the method of Udenfriend (Table 1). The enzymic system

Table 1

Activity of phenylalanine hydroxylase in crude extracts

Composition of the incubation mixture: L-phenylalanine, 6 μ moles; NADH_2 , 1.65 μ mole; nicotinamide, 15 μ moles; enzymic extract, 1.5 ml.; 0.01 M-phosphate buffer, pH 7.0, to final vol. of 3.75 ml. Incubation: 120 min. at 25°, with shaking. Tyrosine was determined in 1.8 ml. samples taken at 0 time and after 120 min., after deproteinization with 0.85 ml. of 15% TCA. Mean values from 1-3 experiments are given. The amount of protein in the samples: *C. euphorbiae* caterpillar 21 mg., pupa 24 mg.; *B. mori* caterpillar 13 mg., pupa 33 mg.; *E. Kühniella* caterpillar 24 mg.; *D. morosus* 7.5 mg.; carp liver 25 mg.; frog liver 24 mg.; pigeon liver 51 mg.; rat liver 65 mg.

Animal	Tyrosine formed (μ moles/100 mg. protein/120 min.)
<i>Celerio euphorbiae</i> caterpillar	1.1
pupa	0
<i>Bombyx mori</i> caterpillar	0.3
pupa	0
<i>Ephestia Kühniella</i> caterpillar	7.2
<i>Dixippus morosus</i> young instar form	10.0
Carp, liver	1.0
Frog, liver	0.4
Pigeon, liver	1.9
Rat, liver	1.4

Table 2

Purification of phenylalanine hydroxylase from caterpillars

Composition of the incubation mixture: L-phenylalanine, 2 μ moles; NADH₂, 3 μ moles; rat liver cofactor, 0.3 ml.; sheep liver enzyme, 0.5 ml. (13 mg. protein); crude or purified extract tested, 0.3 - 0.5 ml.; 1 M-phosphate buffer, pH 6.8, 200 μ moles; final volume 2.0 ml. Incubation: 30 min. at 37°. Tyrosine was determined in 1.8 ml. samples after deproteinization with 0.85 ml. of 15% TCA. Mean values from 2 - 3 determinations are given.

Activity is expressed as μ moles tyrosine formed/100 mg. protein/30 min. The amount of protein in the crude samples: *C. euphorbiae*, 3 mg.; *B. mori*, 2.65 mg.; rat liver, 15.85 mg. The amount of protein in purified samples: *C. euphorbiae*, 0.36 mg.; *B. mori*, 4.75 mg.; *Calliphora*, 3.5 mg.; rat liver, 2.55 mg.

Animal	Crude extract	Purified preparation
<i>Celerio euphorbiae</i>	1	70
<i>Bombyx mori</i>	0.3	1.6
<i>Calliphora</i>	—	2.4
Rat, liver	0.14	9

Table 3

Activity of phenylalanine hydroxylase in C. euphorbiae caterpillar in the presence of dihydrofolic acid

Composition of the incubation mixture and the amounts of protein in the purified extracts, as in Table 2. The results are expressed as μ moles of tyrosine formed/100 mg. protein/30 min.

Sample	<i>C. euphorbiae</i> caterpillar	Rat liver
Whole sample	70	9
NADH ₂ omitted	0	0
Enzymic fraction II omitted	0	0
Natural cofactor omitted	0	0
Natural cofactor omitted, 0.1 ml. dihydrofolic acid added	30	7.3

transforming phenylalanine to tyrosine was found in the livers of all the vertebrates tested. The system was also present in the larval forms of all insects studied, but it was not found in the diapausing pupae of *C. euphorbiae* and *B. mori*. The activity of the enzyme in *D. morosus* and in *E. Kühniella* larvae was several times higher than in *C. euphorbiae* caterpillar and in the livers of vertebrates. Among the vertebrates tested, the lowest activity was found in frog liver.

The hydroxylase (enzyme I) from *C. euphorbiae* caterpillar and from rat liver was purified 70-fold while that from *B. mori* caterpillar only 5-fold (Table 2).

The activity of the purified preparations of phenylalanine hydroxylase from *C. euphorbiae* caterpillar is about 8 times as high as in preparations from rat liver. In preparations from caterpillars of *Calliphora* and *B. mori* the activity was about 1/4 that found in rat liver preparations.

The experiments shown in Table 3 indicate that the purified phenylalanine hydroxylase (enzyme I) from *C. euphorbiae* caterpillar, similarly as that from rat liver, was active only when all components of the incubation system were present, and inactive when any one of them was omitted. If dihydrofolic acid was used instead of the natural cofactor, the enzymic activity was lower by 60% in the caterpillar, and by 20% in rat liver preparations.

DISCUSSION

The obtained results indicate the presence in insect tissues and in the livers of carp, frog and pigeon, of an enzymic system which catalyses the hydroxylation of phenylalanine to tyrosine. The mechanism of the reaction is similar to that demonstrated by Kaufman in mammals. Crude extracts in the presence of NADH₂ hydroxylated phenylalanine; the purified hydroxylase (enzyme I) required for its activity tetrahydropteridine dehydrogenase (enzyme II), the natural cofactor, and NADH₂. The experiments in which the natural cofactor was substituted by dihydrofolic acid indicate that in insects, similarly as in mammals, the pteridine derivative is the cofactor in this reaction.

The demonstration that in insects phenylalanine hydroxylase is present only in larval forms and is absent from pupae indicates the disappearance of tyrosine synthesis during metamorphosis. The results of Karlson and our earlier experiments indicate that at the same time the enzymes catabolising tyrosine also disappear. Sekeris & Karlson [15] reported that aminotransferase and tyrosine decarboxylase, while present in the caterpillar, are absent from the pupa of *Calliphora*. The absence or very low activity of these enzymes was also demonstrated in this laboratory in the pupa of *C. euphorbiae* [1, 2].

SUMMARY

1. Phenylalanine hydroxylase was found to be present in caterpillars of some species of Lepidoptera and in the larval instars of *Dixippus morosus*, as well as in the livers of carp, frog and pigeon.

2. In pupae of *Celerio euphorbiae* and *Bombyx mori* the enzyme was not found.

3. In experiments with purified preparations it was demonstrated that in *C. euphorbiae* caterpillar the mechanism of phenylalanine hydroxylation is similar to that found in mammals.

REFERENCES

- [1] Belżeczka K., Laskowska T. & Mochnacka I. - *Acta Biochim. Polon.* 9, 55, 1962.
- [2] Belżeczka K., Laskowska T. & Mochnacka I. - *Acta Biochim. Polon.* 9, 381, 1962.
- [3] Bricteux-Grégoire S., Verly W. & Florkin M. - *Nature* 177, 1237, 1956.
- [4] Ceriotti G. & Spandrio L. - *Biochem. J.* 66, 607, 1957.
- [5] Fukuda T. - *Nature* 177, 429, 1956.
- [6] Futterman S. - *J. Biol. Chem.* 228, 1031, 1957.
- [7] Kaufman S. - *J. Biol. Chem.* 226, 511, 1957.
- [8] Kaufman S. - *Fed. Proc.* 16, 203, 1957.
- [9] Kaufman S. - *J. Biol. Chem.* 230, 931, 1958.
- [10] Kaufman S. - *Biochim. Biophys. Acta* 27, 428, 1958.
- [11] Kaufman S. - *J. Biol. Chem.* 234, 2677, 1959.
- [12] Kaufman S. - *J. Biol. Chem.* 236, 804, 1961.
- [13] Kaufman S. & Levenberg B. - *J. Biol. Chem.* 234, 2683, 1959.
- [14] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* 2, 279, 1955.
- [15] Sekeris C. & Karlson P. - *Biochim. Biophys. Acta* 62, 103, 1962.
- [16] Udenfriend S. & Cooper J. - *J. Biol. Chem.* 194, 503, 1952.

HYDROKSYLACJA FENYLOALANINY U OWADÓW I NIEKTÓRYCH KRĘGOWCÓW

Streszczenie

1. Stwierdzono obecność hydroksylazy fenyloalaninowej u gąsienic kilku gatunków owadów i młodocianej postaci *Dixippus morosus* oraz w wątrobie karpia, żaby i gołębia.

2. Nie znaleziono badanego enzymu u poczwerek *Celerio euphorbiae* i *Bombyx mori*.

3. W badaniach z oczyszczonymi preparatami wykazano, że mechanizm hydroksylacji u gąsienicy *C. euphorbiae* zachodzi podobnie jak u ssaków.

Received 19 December 1963.

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THE EFFECT OF 2-DEOXY-D-GLUCOSE ON AEROBIC AND ANAEROBIC GLYCOLYSIS IN NEOPLASTIC CELLS AND TISSUES

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Earlier experiments on 2-deoxy-D-glucose suggested the possibility of utilizing this compound as a cytostatic factor. Deoxyglucose inhibits glycolysis in yeast and some animal tissues [13, 14, 10] and growth of cells *in vitro* [2]. Woodward & Hudson [14] demonstrated the inhibiting effect of deoxyglucose on glycolysis in the Flexner-Jobling carcinoma and on Walker 256 carcinosarcoma, and Nirenberg & Hogg [7] observed inhibition of glycolysis in Ehrlich ascites tumour cells. Inhibition of growth of the Crocker rat sarcoma [9] was also observed as well as prolongation of survival time of mice with lymphoid leukemia L-1210 [5]. Still, the mechanism of inhibition by deoxyglucose of the growth and metabolism of cells is not clear.

The aim of the present work was to study the effect of 2-deoxy-D-glucose on aerobic and anaerobic glycolysis in various neoplastic cells. Glucose consumption and lactic acid formation were examined.

MATERIALS AND METHODS

Special reagents. Lactic acid dehydrogenase, glucose oxidase, peroxidase, NAD, and other organic reagents were Boehringer, Mannheim, products. Perchloric acid used for deproteinization was obtained from Merck (Darmstadt), and 2-deoxy-D-glucose from L. Light (Colnbrook, England).

Materials. Three animal tumours were studied: Ehrlich ascites tumour cells, mammary transplantable carcinoma, and mammary spontaneous carcinoma. As carriers of tumours, mice of the inbred strain DBA/2 were used. Both a few mice of this strain and the tumour samples were kindly given by Dr. J. Kieler from the Fibiger Laboratorium, Copenhagen, and were further propagated in our laboratory. For experiments, slices from solid tumours on 14th-20th day of growth and Ehrlich

ascites tumour cells on 7th-10th day of growth, freshly withdrawn from the peritoneal cavity, were used.

Assays. About 150 mg. of slices or about 3×10^7 Ehrlich ascites tumour cells were incubated in a Warburg vessel with 2.0 ml. of the Krebs-Ringer phosphate solution, pH 7.4, containing 10 mM-glucose. Incubation time was 2 hr. at 37° with constant shaking. Deoxyglucose was applied in 5, 10 and 20 mM concentrations. As controls, samples containing deoxyglucose without glucose were used. Aerobic glycolysis was studied in an atmosphere of air and for anaerobic glycolysis the gas phase was nitrogen. At zero time and after 2 hr. of incubation, 0.1 ml. samples were taken for glucose determination and 0.2 ml. samples for lactic acid determination. The latter compound was assayed by the lactic acid dehydrogenase method after Horn & Bruns [3]. To 0.2 ml. of the incubation medium 1.0 ml. of 6% perchloric acid was added; after centrifugation, to 0.1 ml. of the supernatant were added: 2.0 ml. of 0.5 M-glycine buffer, pH 9, containing 0.4 M-hydrazine; 0.03 ml. of lactic acid dehydrogenase (2 mg. protein/ml.) and 0.2 ml. of 0.027 M-NAD. The extinction was measured at 340 m μ in a Hilger spectrophotometer.

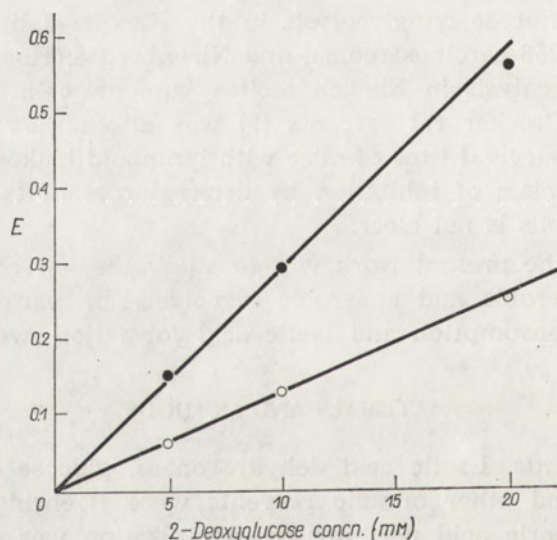


Fig. 1. Oxidation of 2-deoxyglucose by glucose oxidase. (○), Deoxyglucose; (●), glucose.

Glucose was determined by the glucose oxidase method after Hugget & Nixon [4]. To 0.1 ml. of the incubation medium 1.0 ml. of 3.3% perchloric acid was added; after centrifugation, to 0.1 ml. of the supernatant were added 2.5 ml. of a solution containing in 0.12 M-phosphate buffer, pH 7, peroxidase (40 μ g./ml.), glucose oxidase (250 μ g./ml.) and o-dianizidine·HCl (66 μ g./ml.). The extinction was measured at 430 m μ on a Coleman photocolorimeter.

It was found that glucose oxidase preparation (Boehringer, Mannheim) used, oxidized also 2-deoxyglucose, about 43% in comparison to glucose (Fig. 1). On paper chromatography, both Light and Sigma deoxyglucose preparations gave single spots. For calculations of glucose utilization determined by glucose oxidase the values corresponding to the oxidation of deoxyglucose present in the incubation mixture, were taken into account.

In some experiments, for deoxyglucose determination the colorimetric method of Dische, in which glucose gives no colour reaction, in the modification of Seibert [8] was used. No utilization of 2-deoxyglucose by the tumours tested was observed by this method.

RESULTS AND DISCUSSION

In all three tumours tested, aerobic glycolysis (Table 1) determined by the consumption of glucose and formation of lactic acid, was inhibited by deoxyglucose. This is in agreement with the reported effect of deoxyglucose on other neoplastic material [14, 7]. The inhibition was observed

Table 1

The effect of 2-deoxy-D-glucose on the rate of aerobic glycolysis in tumours

The composition of the incubation medium is given under Methods. The results are expressed as median values, \pm confidence intervals, calculated from the range and confidence factors [1]. The number of animals studied is given in parentheses.

Tumour	2-Deoxy-glucose concn. (mM)	Glucose consumption	Inhibition (%)	Lactic acid formation	Inhibition (%)
		(μ moles/10 ⁶ cells/2 hr.)		(μ moles/10 ⁶ cells/2 hr.)	
DBA/2 Ehrlich ascites tumour cells	Nil	0.32 \pm 0.07 (9)		0.63 \pm 0.14 (9)	
	5	0.31 \pm 0.07 (9)		0.57 \pm 0.16 (9)	
	10	0.24 \pm 0.06 (9)	25.0	0.54 \pm 0.18 (10)	14.3
	20	0.12 \pm 0.06 (9)	62.5	0.44 \pm 0.11 (9)	30.2
		(μ moles/100 mg. wet wt./2 hr.)		(μ moles/100 mg. wet wt./2 hr.)	
DBA/2 mammary carcinoma transplantable	Nil	4.09 \pm 0.43 (10)		7.39 \pm 0.63 (10)	
	5	3.46 \pm 0.75 (5)		5.15 \pm 1.9 (5)	
	10	1.87 \pm 0.57 (10)	54.3	4.90 \pm 0.34 (10)	33.7
	20	1.32 \pm 0.77 (5)	67.7	2.30 \pm 1.44 (5)	68.9
DBA/2 mammary carcinoma spontaneous	Nil	5.92 \pm 0.79 (9)		8.35 \pm 1.48 (10)	
	10	4.16 \pm 0.89 (8)	29.6	6.59 \pm 1.24 (9)	21.1
	20	3.24 \pm 0.74 (4)	45.2	5.58 \pm 1.17 (4)	33.2

at 10 mM concentration; smaller amounts of deoxyglucose were without effect and greater amounts increased the inhibition. In the two solid tumours the degree of inhibition measured by glucose utilization and lactic acid formation was similar. In Ehrlich ascites tumour cells, however, glucose consumption was inhibited to a greater extent than lactic acid formation.

Under anaerobic conditions, deoxyglucose concentration required to inhibit glycolysis was also 10 mM (Table 2). The only exception was the mammary transplantable carcinoma in which lactic acid formation was, rather unexpectedly, inhibited by 50% already at 5 mM-deoxyglucose concentration, while practically no inhibition of glucose consumption was simultaneously observed.

Table 2

The effect of 2-deoxy-D-glucose on the rate of anaerobic glycolysis in tumours

The composition of the incubation medium is given under Methods. The results are expressed as median values, \pm confidence intervals [1]. The number of animals studied is given in parentheses.

Tumour	2-Deoxy-glucose concn. (mM)	Glucose consumption	Inhibition (%)	Lactic acid formation	Inhibition (%)
		(μ moles/ 10^6 cells/ 2 hr.)		(μ moles/ 10^6 cells/ 2 hr.)	
DBA/2 Ehrlich ascites tumour cells	Nil	0.41 \pm 0.05 (9)		0.81 \pm 0.13 (10)	
	5	0.40 \pm 0.05 (8)		0.82 \pm 0.14 (10)	
	10	0.26 \pm 0.05 (10)	36.5	0.76 \pm 0.14 (10)	6.2
	20	0.23 \pm 0.07 (8)	43.8	0.68 \pm 0.15 (10)	16.0
		(μ moles/100 mg. wet wt. /2 hr.)		(μ moles/100 mg. wet wt. /2 hr.)	
DBA/2 mammary carcinoma trans-plantable	Nil	5.90 \pm 0.61 (10)		11.28 \pm 1.33 (10)	
	5	4.62 \pm 0.76 (5)		5.70 \pm 1.49 (7)	50.0
	10	3.70 \pm 0.91 (9)	37.2	5.51 \pm 0.77 (10)	51.0
	20	2.05 \pm 1.30 (5)	65.3	2.88 \pm 1.31 (7)	74.4
DBA/2 mammary carcinoma spontaneous	Nil	7.86 \pm 1.30 (7)		11.00 \pm 3.67 (4)	
	10	3.35 \pm 0.64 (6)	57.4	5.75 \pm 1.53 (5)	52.1
	20	1.80 \pm 1.39 (4)	77.1	3.66 \pm 2.48 (4)	69.5

In aerobic, similarly as in anaerobic conditions, in Ehrlich ascites tumour cells glucose consumption was inhibited to a greater extent than lactic acid formation, but so far we are unable to explain this difference. It could be interpreted as indicating the presence of a dual mechanism of deoxyglucose activity. One of these mechanisms would be involved

in the hexokinase reaction or the transport of glucose through the cell membrane, while the second one would be involved in further steps of the glycolytic chain. Such an interpretation would be in agreement with the assumption of Wick *et al.* [11, 12] and Nirenberg & Hogg [7]. According to these authors the effect of deoxyglucose consists in the inhibition of glucose phosphate isomerase and possibly also of hexokinase. Wick *et al.* [6, 11, 12] suggested that the effect of deoxyglucose is connected with the transport of glucose through the cell membrane and that there might be a competition between deoxyglucose and glucose for the sugar transport system.

The authors express their gratitude to Prof. Dr. Irena Mochnacka for help in the preparation of the manuscript. Technical assistance of Miss Danuta Kacprzak and Miss Teresa Nowicka is appreciated.

SUMMARY

The effect of 2-deoxyglucose on aerobic and anaerobic glycolysis was studied on three experimental tumours. Both glucose consumption and lactic acid formation were inhibited, and the effect increased with deoxyglucose concentration.

REFERENCES

- [1] Dean R. B. & Dixon W. J. - *Analyt. Chem.* **23**, 636, 1951.
- [2] Ely J. D., Tull F. A. & Hard J. A. - *J. Franklin Inst.* **253**, 361, 1952.
- [3] Horn H. D. & Bruns F. H. - *Biochim. Biophys. Acta* **21**, 378, 1956.
- [4] Hugget A. G. & Nixon D. A. - *Lancet* **2**, 368, 1957.
- [5] Laszlo J., Landau B., Wight K. & Burk D. - *J. Natl. Cancer Inst.* **21**, 475, 1958.
- [6] Nakada H. I. & Wick A. N. - *J. Biol. Chem.* **222**, 671, 1956.
- [7] Nirenberg M. W. & Hogg J. F. - *Cancer Res.* **18**, 518, 1958.
- [8] Seibert F. B. - *J. Biol. Chem.* **133**, 593, 1940.
- [9] Sokoloff B., Eddy W. H. Saelhof C. C. & Beach J. - *A.M.A. Arch. Pathol.* **59**, 729, 1955.
- [10] Sols A. - *Biochim. Biophys. Acta* **20**, 62, 1956.
- [11] Wick A. N., Douglas R. D. & Toshiko N. M. - *Proc. Soc. Exp. Biol. Med.* **89**, 579, 1955.
- [12] Wick A. N., Drury D. R., Nakada H. I. & Wolfe J. R. - *J. Biol. Chem.* **224**, 963, 1957.
- [13] Woodward G. E. - *J. Franklin Inst.* **254**, 553, 1952.
- [14] Woodward G. E. & Hudson M. T. - *Cancer Res.* **14**, 599, 1954.

WPŁYW 2-DEZOKSY-D-GLIKOZY NA GLIKOLIZĘ KOMÓREK I TKANEK NOWOTWOROWYCH W WARUNKACH TIENOWYCH I BEZTIENOWYCH

Streszczenie

Przebadano wpływ stężeń 2-dezoksyglikozy na glikolizę trzech nowotworów doświadczalnych. Stwierdzono hamujący wpływ 2-dezoksyglikozy na zużycie glikozy i produkcję kwasu mlekowego w warunkach tlenowych i beztlenowych. Wzrastające stężenia 2-dezoksyglikozy pogłębiają efekt hamowania.

Received 20 December 1963.

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METABOLISM OF PHOSPHORUS COMPOUNDS IN MOUSE EMBRYO CELLS GROWN *IN VITRO* AND INFECTED WITH SE POLYOMA VIRUS

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Our previous studies [9] have shown that infection of mouse embryo cells grown *in vitro*, with the oncogenic SE polyoma virus elicits the appearance of features characteristic of neoplastic tissues. Increment in virus titers in the cultures was accompanied by increase in aerobic glycolysis, reduction of endogenous respiration, and increased Crabtree effect, i.e. inhibition of respiratory metabolism on addition of glucose.

Appearance of the Crabtree effect and reduction of respiration in neoplastic material is attributed to changes in the content or distribution of inorganic phosphates and some phosphate esters [11] which, according to the observations of Chance & Hess [3], control the electron transport in normal and neoplastic mitochondria. It appears likely that the increase of the Crabtree effect in the cell after virus infection is related to the synthesis of new virus particles, involving utilization not only of the energetic reserves of the cell but also of phosphorus compounds [1, 13, 14]. To ascertain whether the changes in respiratory metabolism after infection are accompanied by changes in the metabolism of phosphorus compounds, phosphorus fractions were estimated in infected and uninfected cells and conditions affecting assimilation by, and release of inorganic phosphate from, the cells were studied.

MATERIAL AND METHODS

Tissue cultures. The cells were liberated by the action of 0.25% solution of trypsin (Difco) on tissues from embryos of Porton mice, suspended in growth medium in amounts of about 10^5 cells per ml. and transferred to Roux flasks. The growth medium was composed of Earl's salt solution, 0.5% lactalbumin hydrolysate (Nutritional Biochemical Corp., Cleveland, Ohio) and 10% calf serum.

Infection of the cultures with virus. In 48 hr. cell cultures, growth medium was replaced by maintenance medium containing only 3% calf serum. To each Roux flask, containing on the average 7×10^6 cells, was added 1.0 ml. of virus suspension with the infectious titer 10^5 TCID (tissue culture infectious dosis), calculated by the method of Kärber [12].

Preparation of the material for experiments. Parallel virus-infected and uninfected control cultures were prepared for the experiments. The cells were examined daily for 7 days after infection; on the 8th day cytopathogenic changes were so marked that the examinations had to be discontinued. On successive days, the medium from 12 Roux flasks was decanted, the cells remaining on the walls were detached mechanically, combined and suspended in physiological saline solution.

Determination of phosphorus fractions. The cells were washed twice with saline, then suspended in 1% sodium deoxycholate and frozen. According to Kovács [15] the lysis with deoxycholate does not interfere with phosphorus estimation. The phosphorus compounds of the cytolysate were separated by the Schneider method [23] into: acid-soluble fraction, phospholipid, nucleic acid, and protein phosphorus fractions. Phosphorus was determined by the methods of Fiske & Subbarow [7] and Lowry & Lopez [16]. In the acid-soluble fraction inorganic phosphate (P_i) was estimated according to Delory [6]. The results were calculated per 1 mg. of cell dry weight. Dry weight was estimated separately for cells suspended in physiological saline and for cell cytolysates by drying at 105° to constant weight and subtracting the weight of NaCl solute.

Exchange of phosphate between cells and medium. Washed cells were incubated at 37° for 30 - 120 min. in physiological saline solution or in Krebs-Ringer phosphate buffer, pH 7.4, with or without the addition of glucose in the concentration of 10 mM. Inorganic phosphorus was assayed in the incubation medium.

Isotope experiments. Cells were incubated for 30 min. in Krebs-Ringer buffer, pH 7.4, containing 10 mM-glucose and $0.2 \mu\text{C}/\text{ml.}$ of ^{32}P -labelled Na_2HPO_4 . After incubation the cells were centrifuged, washed twice with saline solution and transferred to Krebs-Ringer buffer without the radioisotope; in this buffer the rate of release of [^{32}P]phosphate from the cells was determined. From the centrifuged cells incubated with [^{32}P] Na_2HPO_4 , phosphorus fractions were isolated and their specific activity determined.

Radioactivity measurements. These were made either directly or after mineralization; 0.2 ml. sample was applied on a planchette, dried under an infrared lamp, and the activity measured with a 2π flow-counter (Friesseke, Hoepfner, Germany). The results were expressed as percentages of specific activity of infected cells in relation to that of uninfected cells.

RESULTS

In Table 1 the results of estimation of phosphorus fractions from uninfected cells and from cells on the 4th day after infection, are presented. As it was reported previously, at this time after infection the logarithmic increments in the virus titer and changes in respiratory metabolism had been observed.

Table 1

Phosphorus fractions in mouse embryo cells on the 4-th day after infection with SE polyoma virus, and incorporation of [^{32}P]phosphate

Phosphorus fractions were isolated by the Schneider method [23]. Mean values from 8-14 experiments, \pm S.E., are given. The results are expressed as $\mu\text{g. P/mg. dry cell wt.}$

For isotope experiments the cells were incubated for 30 min. in Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mM-glucose in final concn. and 0.2 $\mu\text{C } ^{32}\text{P/ml.}$ Specific activity was expressed as counts/min./ $\mu\text{g. P,}$ and given as percentage of the sp. act. of control uninfected cells. P values according to Student's t test.

Phosphorus fraction	Uninfected cells	Infected cells	P	Change (%)	Sp. act. (% of uninfected)
Acid-soluble inorganic P	4.61 ± 0.42	3.80 ± 0.35	$=0.13$	-17.6	113
total P	21.29 ± 0.84	15.95 ± 0.87	<0.01	-25.0	116
Phospholipid P	27.4 ± 0.71	23.9 ± 1.69	>0.1	-12.8	121
Nucleic acid P	8.5 ± 0.68	9.7 ± 0.91	>0.1	+14.0	109

On the 4th day after infection in the acid-soluble phosphorus fraction a decrease was observed, both in the total and inorganic P. No statistically significant changes were found in the phospholipid and nucleic acid phosphorus. The results of experiments with inorganic [^{32}P]phosphate showed an increase of specific activity in all fractions (from 9 to 21%) indicating enhanced exchange of phosphorus.

Changes in phosphorus fractions on successive days after infection are given in Table 2. For 24 hr. after inoculation with the virus no changes were observed. Beginning with the 2nd day, the content of the acid-soluble fraction, both P_i and total P, became lower in infected cells and this difference persisted or even increased with time. The content of lipid phosphorus increased at first but beginning with the 4th day also decreased to levels below the control values. Similarly the increase in protein phosphorus was followed by a decrease. The content of nucleic acid phosphorus did not change till the 5th day and then it increased.

Table 2

Changes in phosphorus fractions in mouse embryo cells on successive days after infection with SE polyoma virus

Mean values in $\mu\text{g. P/mg. dry cell wt.}$ are given; in parentheses the number of experiments.

Days after infection	Cells	Acid-soluble		Phospho-lipid P	Nucleic acid P	Protein P
		P _i	total P			
1	Uninfected	5.55	22.05	32.0	7.7	13.1
	Infected	5.90 (2)	22.10 (2)	31.9 (2)	7.4 (3)	15.4 (2)
	Change (%)					+18
2	Uninfected	5.50	20.2	22.4	7.2	—
	Infected	4.17 (3)	13.4 (3)	32.2 (2)	7.4 (2)	—
	Change (%)	—24	—34	+44		
3	Uninfected	4.64	18.0	22.0	6.0	18.0
	Infected	3.57 (4)	11.7 (4)	62.5 (1)	6.2 (2)	17.5 (1)
	Change (%)	—23	—23			
5	Uninfected	3.47	20.8	33.6	12.8	—
	Infected	2.66 (1)	16.1 (2)	25.3 (2)	13.8 ()	—
	Change (%)	—23	—23	—25		
6	Uninfected	3.92	21.4	28.3	11.9	—
	Infected	2.15 (1)	14.6 (1)	19.2 (1)	15.1 (1)	—
	Change (%)	—47	—32	—28	+27	
7	Uninfected	3.40	18.1	25.0	8.8	11.7
	Infected	2.70 (4)	14.2 (4)	16.9 (1)	11.2 (3)	9.5 (2)
	Change (%)	—21	—22	—32	+25	—18

Table 3

The exchange of phosphorus between cells and different media in the course of incubation

Mouse embryo cells, uninfected and on the 6th day after infection with SE polyoma virus, were incubated in the medium indicated in the Table. The content of phosphorus is expressed as $\mu\text{g.P/mg. dry cell wt.}$; increase or decrease of P content is given in parentheses.

Incubation in...	Physiological saline		Physiological saline + glucose (10 mm)		Krebs-Ringer phosphate buffer + glucose (10 mm)	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
Time (min.)						
0	1.33	1.04	1.30	1.50	5.6	5.6
30	1.44 (+0.11)	1.32 (+0.28)	—	1.57 (+0.07)	5.3 (—0.3)	5.6
60	1.60 (+0.27)	1.38 (+0.34)	1.48 (+0.18)	1.73 (+0.23)	5.0 (—0.6)	5.1 (—0.5)
90	1.75 (+0.42)	1.41 (+0.37)	1.60 (+0.30)	1.90 (+0.40)	4.9 (—0.7)	5.0 (—0.6)
120	1.80 (+0.47)	1.41 (+0.37)	1.70 (+0.40)	2.05 (+0.55)	4.52 (—1.1)	4.9 (—0.7)

The exchange of phosphorus between the cells and different media is presented in Table 3. Washed non-infected cells and cells after infection were suspended and incubated in different media, in which inorganic P was determined. Although the differences were not very large, the data in Table 3 indicate an increment of P_i in physiological saline solution during incubation of washed cells. In the case of infected cells, the release was faster. Presence of glucose in the physiological saline solution used for the incubation, retarded the release of P_i into the medium. In phosphate-glucose buffer the uptake of inorganic phosphate was observed; it was somewhat delayed in infected cells.

In Fig. 1 the exchange of ^{32}P between the cells and the medium is illustrated. The mean value obtained from 6 successive determinations,

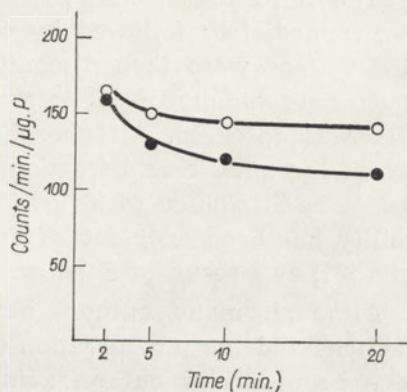


Fig. 1. The exchange of phosphorus between cells and medium. Mouse embryo cells preincubated for 30 min. in Krebs-Ringer phosphate buffer containing 10 mM-glucose and 0.2 μ C/ml. of ^{32}P , were washed twice with saline solution and suspended in Krebs-Ringer phosphate buffer without glucose and ^{32}P . Specific activity was determined after 20 min. incubation of (●), cells infected with SE polyoma virus; (○), uninfected cells.

of decrease of activity in infected cells after 20 min. incubation, in relation to uninfected cells, was $31.5\% \pm 2.1$ (S.E.). From the infected cells, phosphorus was released into the medium faster than from uninfected ones.

DISCUSSION

Our previous [9] and present results indicate that in the metabolism of cells infected with the SE polyoma virus, three phases may be discerned related to a certain extent to the phases of virus development. In the first phase, lasting up to 24 hr., during which the virus was not yet detectable, no changes in the content of phosphorus compounds in phosphorus fractions were found, notwithstanding that already at this time some reduction in oxygen consumption and appearance of aerobic glycolysis have been observed [9]. At the beginning of the second phase, distinct changes took place. In the acid-soluble fraction, inorganic and total phosphorus were diminished already on the second day after infection, while lipid phosphorus increased. At the same time virus titers in

the culture fluid began to increase. Considering that the cells were not infected with an excess of the virus, this phase may be regarded as corresponding to the release of the virus from only a part of the infected cells, while the remaining cells were in the stage of virus reinfection or of intensive intracellular synthesis of the virus. The increase in lipid and protein phosphorus observed at the beginning of this phase might be an indication of enhanced intra-cellular synthesis; later, at the time of further release of the virus, a decrease of these fractions was observed. In previous experiments, aerobic glycolysis was increasing throughout the second phase proportionally to the increasing virus titer, and a further decrease of endogenous respiration and an increase of the Crabtree effect, were observed.

The third phase, observed on the 6th and 7th days after infection, was immediately followed by cytopathogenic changes in the cells, and observations were then discontinued. During this phase, respiration as well as aerobic glycolysis decreased and no further increase in the virus titer was observed. All phosphorus fractions remained at a low level except the nucleic acid fraction which increased. However, this increase cannot be attributed to formation of virus nucleoproteins until the possibility has been excluded of an increase in the number of polyploid cells [21] as a result of impairment of cellular mitosis by the virus.

Although many authors have studied the changes in phosphorus fractions and the incorporation of ^{32}P in virus-infected cells, few studies have been carried out on animal cells. In most of these, HeLa cells infected with poliomyelitis viruses [18, 17] or homogenates of brain tissue infected with Theiler's virus [19, 20] were used. Studies on tissues infected with other viruses, e.g. of influenza [4] or adenoviruses [8] are rare. Although no direct comparison can be made between these results, observations made under different conditions bring out the same phenomena: initial stimulation of protein and phospholipid synthesis by the virus followed by their decrease during the release of the virus. Significant increase in the RNA and DNA fractions of the cells [22, 5, 8] has also been found, and attention has been called to changes in the permeability of the cell membranes and in assimilation of P_i by virus-infected cells [2].

Numerous studies show that penetration of phosphorus into the cell is an active process [10, 24]. A faster exchange of phosphorus between the medium and the infected cell may be due to greater permeability and impaired retention of phosphate. This latter phenomenon is probably connected with the reduced respiration and oxidative phosphorylation. The presented results are in agreement with the assumption that in the virus-infected cell changes in respiratory metabolism are accompanied by changes in the metabolism of phosphorus compounds.

SUMMARY

In mouse embryo cells grown *in vitro* and infected with SE polyoma virus a decrease of acid-soluble phosphorus content was observed. The phospholipid and protein P fractions which were higher than the controls in the first phase, later diminished in proportion to the increase in virus titer. Only the nucleic acid P increased. Experiments with ^{32}P showed increased exchange of the phosphorus, and changes in permeability to phosphorus compounds of infected cells.

REFERENCES

- [1] Ackermann W. W. - *Bact. Rev.* **22**, 223, 1958.
- [2] Becker Y., Grossowicz N. & Bernkopf H. - *Proc. Soc. Expt. Biol. Med.* **97**, 77, 1958.
- [3] Chance B. & Hess B. - *J. Biol. Chem.* **234**, 2413, 1959.
- [4] Cohn Z. A. - *Proc. Soc. Exptl. Biol. Med.* **79**, 566, 1952.
- [5] Contreras G., Toha J. & Ohlbaum A. - *Biochim. Biophys. Acta* **35**, 268, 1959.
- [6] Delory G. E. - *Biochem. J.* **32**, 1161, 1938.
- [7] Fiske C. H. & Subbarow Y. - *J. Biol. Chem.* **66**, 375, 1925.
- [8] Ginsberg H. S., *Perspectives in Virology* (M. Pollard, ed.) vol. II, p. 58. Burghess Publ. Co, Minneapolis 1961.
- [9] Gumińska M., Skarżyński B. & Porwit-Bóbr Z. - *Acta Biochim. Polon.* **10**, 163, 1963.
- [10] Holzer H. - *Biochim. Z.* **324**, 144, 1953.
- [11] Ibsen K. H. - *Cancer Res.* **21**, 829, 1961.
- [12] Irwin J. O. & Cheeseman E. A. - *J. Hyg.* **39**, 574, 1959.
- [13] Korbecki M. - *Postępy Hig. Med. Dośw.* **15**, 497, 1961.
- [14] Kovács E. - *Experientia* **12**, 153, 1961.
- [15] Kovács E. - *Arch. Biochem. Biophys.* **76**, 546, 1958.
- [16] Lowry O. H. & Lopez J. A. - *J. Biol. Chem.* **162**, 421, 1946.
- [17] Maassab H. F., Loh Ph. C. & Ackermann W. W. - *J. Exp. Med.* **106**, 641, 1957.
- [18] Miroff G., Cornatzer W. C. & Fischer R. G. - *J. Biol. Chem.* **228**, 255, 1957.
- [19] Moldave K. - *J. Biol. Chem.* **210**, 343, 1954.
- [20] Rafelson M. E., Winzler R. J. & Pearson H. E. - *J. Biol. Chem.* **181**, 583, 1949.
- [21] Salzman N. P. - *Biochim. Biophys. Acta* **31**, 158, 1959.
- [22] Salzman N. P. & Lockart R. Z., Jr. - *Biochim. Biophys. Acta* **32**, 572, 1959.
- [23] Schneider W. C. - *J. Biol. Chem.* **161**, 293, 1945.
- [24] Shacter B. - *Arch. Biochem. Biophys.* **57**, 387, 1955.

METABOLIZM ZWIĄZKÓW FOSFOROWYCH W EMBRIONALNYCH
KOMÓRKACH MYSICH HODOWANYCH *IN VITRO* I ZAKAŻONYCH
WIRUSEM SE POLYOMA

Streszczenie

W embrionalnych komórkach mysich hodowanych *in vitro* i zakażonych wirusem SE polyoma stwierdzono w okresie uwalniania wirusa do środowiska spadek kwasorozpuszczalnej frakcji fosforowej. Frakcja fosfolipidowa i fosfoproteinowa, wyższa w pierwszym okresie w porównaniu z kontrolną, spadała proporcjonalnie do przyrostu miana wirusa. Badania izotopowe wykazały zwiększoną wymianę fosforu badanych frakcji oraz zwiększoną przepuszczalność dla związków fosforowych.

Received 20 December 1963.

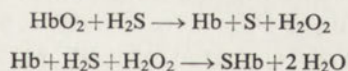
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STUDIES ON SULPHAEMOGLOBIN FORMATION

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The action of hydrogen sulphide or sulphides on oxyhaemoglobin produces sulphaemoglobin. In spite of extensive research carried out since the description of this compound in 1886 by Hoppe-Seyler [11], its exact structure is unknown. According to Michel [14] a molecule of sulphaemoglobin possesses four sulphur atoms, and its molecular weight is the same as that of haemoglobin. Most investigators agree that the α -methene carbon binds the additional atom of sulphur [14, 17, 16, 7] but according to Lemberg & Legge [13] sulphur may form a linkage between the iron of haem and histidine of globin.

The reaction by which sulphaemoglobin is formed is not yet fully elucidated. Thus far, a complete conversion of oxyhaemoglobin into sulphaemoglobin has never been obtained even when a large excess of hydrogen sulphide was applied [13]. It is known that the reaction of haemoglobin with hydrogen sulphide does not occur in the absence of oxygen but the addition of one drop of 3% solution of H_2O_2 causes immediate appearance of the characteristic absorption band at 620 m μ [14, 6]. Since hydrogen peroxide may be formed during oxidation of hydrogen sulphide, Michel [14] has proposed the following scheme of the reaction leading to formation of sulphaemoglobin (SHb):



Although not all authors agree with this scheme [17], the suggestion of Michel is supported by the observation that formation of sulphaemoglobin *in vitro* is accelerated by reducing substances derived from aromatic amines [2, 13] such as phenylhydrazine which in reaction with oxyhaemoglobin gives rise to peroxide groups [18]. Sulphaemoglobin may appear in the human blood after prolonged administration of analgetic drugs, especially phenacetin and acetanilide [4], leading to a pathological condition known as sulphaemoglobinemia. We have pre-

viously discussed the mechanism of the development of sulphaemoglobinemia [9, 8].

In the present work the effect of various factors on the reaction of oxyhaemoglobin with hydrogen sulphide was studied.

MATERIALS AND METHODS

Material. Intact human erythrocytes or freshly made haemolysates were used. Erythrocytes from citrated venous blood were washed three times with physiological saline solution and after each centrifugation the layer containing platelets and leucocytes was discarded. The sedimented erythrocytes were added with an equal volume of a mixture of 0.9% NaCl and 0.12 M-phosphate buffer of pH 7.4 (1:3, v/v); thus a 50% suspension was obtained. The haemolysates were prepared by adding 1 ml. of packed erythrocytes to 50 ml. of bidistilled water. From the haemolysates, stroma was separated by centrifuging at 8000 r.p.m. for 20 min. and the supernatant was dialysed against 0.03 M-phosphate buffer, pH 7.4, for 24 hr. at 4°. Just before the experiment, the dialysed haemolysate was diluted with an equal volume of the same buffer, and haemoglobin concentration was determined spectrophotometrically. In different samples the concentration of haemoglobin ranged from 0.1 to 0.2 mm.

Freshly prepared solutions of hydrogen sulphide in bidistilled water were used, hydrogen sulphide being obtained from barium sulphide and hydrochloric acid. In the experiments with intact erythrocytes, hydrogen sulphide was dissolved in 0.9% NaCl. The concentration of hydrogen sulphide in the solution was determined by the colorimetric method of Almy [1].

Special reagents. Glutathione (GSH) and phenylhydrazine were products of the BDH Co., Poole, England. L-Cystine, L-cysteine and N-ethylmaleimide (NEM) were products of Schuchardt Co., München; DL-methionine of the Ashe Co., London; cysteinic acid of the Light Co., Colnbrook, England; sodium thiosulphate p.a. and hydroxylamine were Merck, Darmstadt, products. Pharmacopoeial preparations of phenacetin and acetanilide were used. Oxidized glutathione (GSSG) was obtained by oxidation of GSH with iodine and separation on a Dowex-50 (H⁺ form) column. The obtained preparation gave no reaction with nitroprusside and on the ninhydrin-stained chromatograms gave a single spot corresponding to standard GSSG. All the substances were dissolved just before the experiments.

Determination of glutathione. The content of GSH in the samples was determined by the method of Grunert & Phillips [10].

Assays on haemolysates. Sulphaemoglobin formation was assessed in the following way. To 3 ml. of the haemolysate 2 ml. of bidistilled water

was added in the blank test, and 1 ml. of water and 1 ml. of hydrogen sulphide solution in the proper sample. The tubes were immediately stoppered, mixed, and the contents were transferred to closed cuvettes; the increment in extinction at 620 m μ of the proper sample was measured with the Hilger Uvispec spectrophotometer against the blank at intervals of 1 - 2 min. for 10 - 30 min.

The procedure for assessing the effect of the tested compounds was as follows. To four test tubes, each containing 3 ml. of the haemolysate, were added, respectively: (1), 2 ml. of water; (2), 1 ml. of water and 1 ml. of H₂S solution; (3), 1 ml. of water and 1 ml. of the solution of the tested compound; (4), 1 ml. of the solution of the tested compound and 1 ml. of H₂S solution. The tested compound was mixed with the haemolysate not earlier than 5 min. before the addition of H₂S solution. The H₂S solution was added simultaneously to tubes no. 2 and 4. The contents of all tubes were transferred to four cuvettes, and the increment in extinction in the proper samples was measured against the blanks. Two blanks (tubes no. 1 and 3) were necessary because some of the tested compounds, e.g. phenylhydrazine, may form with haemoglobin some derivatives with unspecific absorption spectra. Since the absorption spectrum of choleglobin closely resembles that of sulphaemoglobin, its presence might have affected the results; therefore after absorption measurements the samples were treated with carbon monoxide and sodium dithionite according to Lemberg & Legge [13] and examined in the Hartridge reversion spectroscopy; no choleglobin was found in any of the samples.

Assays on intact erythrocytes. In these experiments, 0.2 - 0.3 ml. of 50% erythrocyte suspension was incubated with a solution of hydrogen sulphide in 0.9% NaCl. The erythrocytes were then sedimented by centrifugation, washed with 0.9% NaCl, haemolysed in bidistilled water, and the extinction was measured at 620 m μ .

RESULTS

Data presented in Table 1 indicate that the rate of sulphaemoglobin formation is higher in the haemolysate than in intact erythrocytes.

The effect of stroma on haemolysates was studied in experiments in which the haemolysate of erythrocytes (1:50) was divided into two parts. One of them was centrifuged for 20 min. at 8000 r.p.m., the stroma thus obtained was added to the other, uncentrifuged part, and in both the rate of sulphaemoglobin formation was estimated. The results presented in Fig. 1 indicate that the addition of stroma accelerates the reaction of haemoglobin with hydrogen sulphide. On the other hand, the rate of the reaction was slightly slower on addition of serum of the blood from which the haemolysate was prepared.

Table 1

Sulphaemoglobin formation rate in intact erythrocytes and in the haemolysate

To 0.2 ml. of erythrocyte suspension were added either 8 ml. of water and 1 ml. of 0.06 M-phosphate buffer, pH 7.4, or 8 ml. of 0.9% NaCl solution and 1 ml. of 0.06 M-phosphate buffer. To each tube 1 ml. of H₂S solution (7 μ moles) in 0.9% NaCl was added, then the tubes were incubated for 10 min. at room temp. In the haemolysate the extinction was measured against water at 620 m μ . The erythrocytes were centrifuged, haemolysed with 8 ml. of water, then 1 ml. of phosphate buffer and 1 ml. of 0.9% NaCl solution were added, and the extinction at 620 m μ was determined. Under the heading "Time" is given the interval between the addition of H₂S and measurement of extinction.

Sample	E _{620 mμ}	Time (min.)
Haemolysate	0.63	14
Erythrocytes	0.33	24

The effect of phenacetin, acetanilide, phenylhydrazine and hydroxylamine on the formation of sulphaemoglobin in dialysed haemolysates is presented in Table 2. It may be seen that phenacetin, phenylhydrazine and hydroxylamine accelerated the reaction. This effect was most pro-

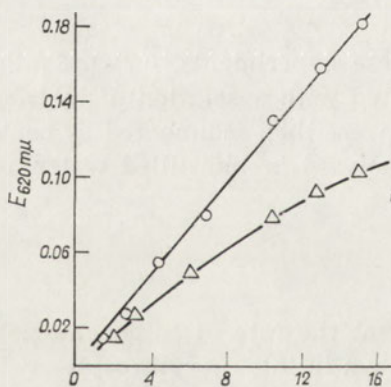


Fig. 1

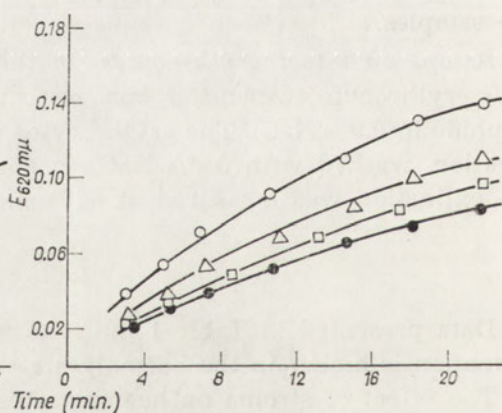


Fig. 2

Fig. 1. Effect of stroma on sulphaemoglobin formation in the haemolysate. Each sample contained 0.15 mM-solution of haemoglobin and 0.4 mM H₂S. (○), Haemolysate enriched in stroma; (Δ), haemolysate without stroma.

Fig. 2. Effect of GSH on sulphaemoglobin formation in the haemolysate. Each sample contained 0.13 mM-solution of haemoglobin and 0.48 mM-H₂S. (○), Control, without GSH added; (Δ), 0.02 mM-GSH; (□), 0.1 mM-GSH; (●), 0.2 mM-GSH.

nounced at pH 7.4. The effect of phenacetin was more noticeable at low concentrations of hydrogen sulphide (less than 0.2 mM), and that of phenylhydrazine was most distinct during the first 2 min. of incubation. The effect of these substances was observed also in non-dialysed haemolysates enriched in stroma, and in intact erythrocytes.

Table 2

Effect of phenacetin, acetanilide, phenylhydrazine and hydroxylamine on the rate of sulphaemoglobin formation in dialysed haemolysates

Final concentration of H_2S in the sample was 0.6 mM, that of the added substance 0.2 mM. Incubation 15 min. For details see Methods. Twelve experiments were performed and the results of a typical one are presented.

Addition	$E_{620\text{ m}\mu}$	Effect (%)
None	0.156	32
Phenacetin	0.204	
None	0.151	5
Acetanilide	0.158	
None	0.159	29
Phenylhydrazine	0.205	
None	0.152	49
Hydroxylamine	0.226	

Table 3

Effect of GSH on sulphaemoglobin formation in erythrocytes

Suspensions of erythrocytes were preincubated for 12 hr. as indicated in the Table. After centrifugation and washing, the content of GSH was determined and 0.3 ml. of 50% erythrocyte suspension was incubated for 10 min. with 1 ml. of H_2S solution (9 μ moles). In the haemolysate, sulphaemoglobin was determined.

Erythrocytes preincubated with	Content of GSH in erythrocyte suspension (μ mole/ml.)	$E_{620\text{ m}\mu}$
Glucose, 6.8 mM	0.61	0.53
Physiological saline solution	0.53	0.65
Phenacetin, 0.5 mM	0.40	0.69
Phenylhydrazine, 0.5 mM	0.15	0.81

It has long been known that phenylhydrazine accelerates the oxidation of glutathione in erythrocytes, and this phenomenon constitutes the basis of Beutler's GSH stability test [3] for detecting the deficiency of glucose-6-phosphate dehydrogenase in erythrocytes. Also in our experiments, the erythrocytes incubated with phenacetin contained less GSH than those incubated without phenacetin. This suggested the participation of glutathione in sulphaemoglobin formation. Therefore suspensions of erythrocytes were preincubated for 12 hr. at room temp. with glucose, phenacetin or phenylhydrazine. Unequal amounts of GSH in the samples were found (Table 3). The erythrocytes were washed, incubated for 10 min. with hydrogen sulphide, and the amount of sulphaemoglobin was determined. The results suggest that at higher GSH concentrations the formation of sulphaemoglobin is inhibited. This was confirmed in another experiment in which the erythrocytes were preincubated with *N*-ethylmaleimide to block the SH compounds [12]. In NEM-treated erythrocytes, in which the amount of GSH was practically nil, the rate of sulphaemoglobin formation was markedly increased (Table 4).

Table 4

Formation of sulphaemoglobin in erythrocytes in which the SH compounds were blocked by N-ethylmaleimide

Two 0.5 ml. samples of 50% erythrocyte suspension were preincubated for 10 min. with 0.2 ml. of solution of *N*-ethylmaleimide (10 μ moles/ml.). Then the erythrocytes were washed and GSH was determined in one sample while the second one was incubated for 5 min. with 1 ml. of H_2S solution in 0.9% NaCl (5 μ moles), and the amount of sulphaemoglobin was determined. The control samples without NEM were prepared in the same way.

Erythrocytes	Content of GSH in erythrocyte suspension (μ mole/ml.)	$E_{620\text{ m}\mu}$
Control	0.65	0.16
Preincubated with NEM	0.01	0.32

Further data on the effect of glutathione on sulphaemoglobin formation were obtained in experiments with stroma-free, dialysed haemolysate (Fig. 2). It may be seen that GSH already at the concentration of 0.02 mM retarded the reaction, and at 0.2 mM-GSH the effect was doubled. Other natural SH compounds, as cysteine and ergothioneine, had a similar effect (Table 5).

In analogous experiments the effect of oxidized glutathione and cystine in 0.1 mM concentrations was also observed but it was about

Table 5

Inhibition of sulphaemoglobin formation in the presence of SH compounds

All the samples contained 3 ml. of dialysed haemolysate (0.12 mM-HbO₂) in 0.03 M-phosphate buffer, pH 7.4. Five min. after the addition of the compound tested, 1 ml. of H₂S solution was added, and after 10 min. incubation at room temp. the extinction was measured.

Addition (0.2 mM-concn.)	E _{620 mμ}	Inhibition (%)
None	0.245	
Cysteine	0.104	59.0
GSH	0.139	43.2
Ergothioneine	0.214	12.5

three times smaller than that of reduced glutathione or cysteine. The inhibiting effect of these bisulphides may be explained by their partial conversion to thiols in the presence of hydrogen sulphide. On the other hand, methionine, cysteinic acid and thiosulphate had no effect on the formation of sulphaemoglobin.

When phenacetin and GSH in concentrations of 0.1 mM were added simultaneously to the haemolysate, the formation of sulphaemoglobin was inhibited in the same degree as by GSH alone. Therefore the effect

Table 6

Effect of phenacetin, acetanilide, phenylhydrazine and hydroxylamine on GSH oxidation in the haemolysate

Each sample contained 5 ml. of the haemolysate (0.1 mM-HbO₂), 1 μmole GSH (0.2 mM), and the tested compound. In the controls directly, and in the proper samples after 2 hr. of incubation at 37°, protein was precipitated with 1 ml. of 20% trichloroacetic acid, and GSH was determined in the supernatant.

Addition (0.1 mM-concn.)	Incubation (hr.)	Glutathione	
		content (μmole/sample)	decrease (%)
None	0	1.00	
	2	0.91	9
Phenacetin	0	0.99	
	2	0.86	13
Acetanilide	0	1.01	
	2	0.88	13
Phenylhydra- zine	0	0.98	
	2	0.80	18
Hydroxyl- amine	0	0.99	
	2	0.76	23

of phenacetin, acetanilide, phenylhydrazine and hydroxylamine on the rate of oxidation of GSH in the haemolysate was examined (Table 6) and it was found that these compounds accelerated the disappearance of GSH.

DISCUSSION

In 1925 Van den Bergh & Wieringa [2] observed that phenylhydrazine, hydroxylamine and certain other aromatic amines accelerate the reaction of oxyhaemoglobin with hydrogen sulphide. In the present experiments it was demonstrated that also phenacetin, the antipyretic drug causing sulphaemoglobinemia in man, has a similar effect. If the assumption of Michel [14] is correct, it is reasonable to attribute the effect of these substances to formation of hydrogen peroxide during their oxidation in the haemolysate [2, 14, 18]. Direct participation of hydrogen peroxide in the formation of sulphaemoglobin was confirmed by the spectrokinetic studies of Dalziel & O'Brien [6]. In the reaction of oxyhaemoglobin with hydrogen sulphide without addition of phenylhydrazine or related compounds, trace quantities of H_2O_2 necessary for the formation of sulphaemoglobin may arise as the result of autooxidation of hydrogen sulphide [14]. In the presented experiments, the observed inhibition of sulphaemoglobin formation by GSH, cysteine or ergothioneine is probably due to the removal of hydrogen peroxide from the reaction medium. Cohen & Hochstein [5] demonstrated that the amount of GSH in erythrocytes incubated with small amounts of hydrogen peroxide decreases, the effect being more pronounced in erythrocytes deficient in glucose-6-phosphate dehydrogenase. According to Mills [15] erythrocytes possess an enzyme, glutathione peroxidase, which catalyses the oxidation of GSH by hydrogen peroxide. Mills assumed also that the activity of this enzyme is the main factor protecting haemoglobin from conversion into choleglobin, as other SH compounds did not inhibit the formation of choleglobin from oxyhaemoglobin in the presence of ascorbic acid. In the presented experiments, GSH reduced the rate of sulphaemoglobin formation. This might seem to suggest an analogy with the reaction described by Mills, however, in the reaction of haemoglobin with hydrogen sulphide other thiol compounds also inhibited sulphaemoglobin formation and cysteine was even more effective than glutathione.

In view of the high concentration of reduced glutathione in erythrocytes, this compound seems to be a natural inhibitor of the formation of sulphaemoglobin *in vivo*. The data presented in Table 6 indicate that all the substances which accelerate the formation of sulphaemoglobin, increase the oxidation of glutathione in the haemolysate. Presumably, the effect of these substances consists in formation of H_2O_2 .

Sulphaemoglobin is formed more quickly in the haemolysate than in

intact erythrocytes. This may be due to the dilution of GSH and other SH compounds and to the free access of hydrogen sulphide to haemoglobin in the absence of the erythrocyte membrane. On the other hand, stroma was found to accelerate the formation of sulphaemoglobin in the haemolysates.

SUMMARY

The reaction of oxyhaemoglobin with hydrogen sulphide was studied on intact erythrocytes and on haemolysates in the presence of various substances. Phenacetin, phenylhydrazine and hydroxylamine accelerated, while glutathione, cysteine and ergothioneine inhibited the reaction. The SH compounds in erythrocytes appear to participate in the mechanism protecting haemoglobin from conversion to sulphaemoglobin *in vivo*. The presented results support the suggestion that peroxide groups are involved in sulphaemoglobin formation.

REFERENCES

- [1] Almy H. - *J. Am. Chem. Soc.* **47**, 1381, 1925.
- [2] Bergh v. d. A. A. H. & Wieringa A. - *J. Physiol.* **59**, 407, 1925.
- [3] Beutler E. - *J. Lab. Clin. Med.* **49**, 84, 1957.
- [4] Brandenburg R. O. & Smith H. L. - *Am. Heart J.* **42**, 582, 1951.
- [5] Cohen G. & Hochstein P. - *Science* **134**, 1756, 1961.
- [6] Dalziel K. & O'Brien J. R. P. - *Biochem. J.* **67**, 124, 1957.
- [7] Foulkes E. C., Lemberg R. & Purdom P. - *Proc. Roy. Soc. B.* **138**, 386, 1951.
- [8] Frendo J., *Badania nad mechanizmem powstawania sulfhemoglobinemii*. Thesis. Medical School in Kraków, 1963.
- [9] Frendo J. & Koj A. - *Polskie Archiwum Medycyny Wewnętrznej* **32**, 867, 1962.
- [10] Grunert R. R. & Phillips P. H. - *Arch. Biochem.* **30**, 217, 1951.
- [11] Hoppe-Seyler F. - *Centr. med. Wiss.* **436**, 1866.
- [12] Jacob H. S. & Jandl J. H. - *J. Clin. Invest.* **41**, 779, 1962.
- [13] Lemberg R. & Legge J. W., *Hematin Compounds and Bile Pigments*, pp. 490-499, Intersc. Publ., N. York 1949.
- [14] Michel H. O. - *J. Biol. Chem.* **126**, 323, 1938.
- [15] Mills G. C. - *J. Biol. Chem.* **229**, 189, 1957.
- [16] Nichols P. - *Biochem. J.* **81**, 374, 1961.
- [17] Nijveld H. A. - *Rec. trav. chim.* **62**, 293, 1943.
- [18] Rostorfer H. H. & Cormier M. J. - *Arch. Biochem. Biophys.* **71**, 235, 1957.

BADANIA NAD POWSTAWANIEM SULFHEMOGLOBINY

Streszczenie

Badano reakcję oksyhemoglobiny z siarkowodorem w całych erytrocytach i hemolizatach w obecności rozmaitych substancji. Fenacetyna, fenilohydrazyna i hydroksylamina przyspieszają, a glutation, cysteina i ergotioneina hamują reakcję. Związki -SH w erytrocytach wydają się być związane z mechanizmem chroniącym *in vivo* hemoglobinę przed zamianą na sulfhemoglobinę. Przedstawione dane przemawiają za udziałem ugrupowań nadlenkowych w powstawaniu sulfhemoglobiny.

Received 20 December 1963.

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ISOLATION AND PRELIMINARY FRACTIONATION OF URINARY PEPTIDES BY MEANS OF GEL FILTRATION

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Normal daily urine contains about 2 g. of amino acids which may be liberated by acid hydrolysis [14]; some of them are derived from peptide compounds. In recent years a number of peptides from normal and pathological urines have been isolated and characterized [cf. 13]. Normal urine contains small amounts of protein but no simple method exists for separation of peptides from proteins and non-peptide amino acid compounds in the urine. As we have observed that by filtration of urine through Sephadex G-75 gel a good separation of high molecular compounds is obtained [4, 12], this method was used for the isolation and preliminary fractionation of urinary peptides.

MATERIAL AND METHODS

Experiments were carried out on samples of 24-hr. urines of healthy women. To inhibit bacterial growth, toluene was added. The urine contained no sugar as tested with Nylander's reagent, and the sulphosalicylic acid test for protein was negative. Fractionation was carried out by gel filtration on Sephadex G-75 medium and G-25 medium (Pharmacia Co., Uppsala).

Fractionation. Fifty ml. of urine was introduced on a Sephadex G-75 column (3.8 × 40 cm.) equilibrated with phosphate buffer, pH 6.0 and ionic strength 0.10. Elution was carried out with the same buffer, and 10 ml. fractions were collected until the ninhydrin test became negative.

The non-protein fractions (tubes 27 - 40) corresponding to 100 ml. of urine were combined, concentrated under reduced pressure at 40° to the volume of 50 ml. and dialysed (Visking tubing) at 4° against 600 ml. of water, changed three times during 48 hr. The diffusate (FD) was concentrated as above to the volume of 50 ml. and the non-diffusible material (FN) was made up to 100 ml. Both fractions were then separated

on Sephadex G-25 column (2.8×28 cm.), the volumes applied corresponding respectively, to 4 and 80 mg. of total nitrogen. Elution was carried out with bidistilled water, and 8 ml. fractions were collected.

Analysis of the fractions. In the eluates from Sephadex G-75, absorption at $280\text{ m}\mu$ was measured with a Hilger spectrophotometer. In the combined protein fractions, total nitrogen and α -amino nitrogen were determined before and after acid hydrolysis.

One ml. samples of the eluates from the Sephadex G-25 column served for determination of α -amino nitrogen by the ninhydrin method, and 2 ml. samples for sugar estimation by the anthrone method. Fractions exhibiting maximum coloration with ninhydrin and the anthrone reagent were then submitted to paper chromatography before and after hydrolysis. Presence of bound amino acids was determined after hydrolysis with 6 N-HCl at 110° for 24 hr. Sugars bound with peptides were detected after hydrolysis with $1\text{ N-H}_2\text{SO}_4$ for 2 hr. at 110° .

Analytical methods. Sugars were determined by the anthrone method [15]. Total N was assayed by the method of Nessler [3], α -amino nitrogen before and after hydrolysis by the ninhydrin method of Rosen [10]. Before the determination, the samples were treated with 1 N-NaOH , and ammonia was removed by placing them over conc. sulphuric acid under reduced pressure for 24 hr. Circular paper chromatography was done on Whatman no. 1 filter paper; the chromatograms were developed in a system of butan-1-ol - acetic acid - water (144:13:43, by vol.) and stained with acetonetic solution of ninhydrin and isatin [7] and with aniline-phthalate reagent [9].

RESULTS

The fractionation of urine on Sephadex G-75 column is illustrated in Fig. 1. Peaks I and Ia contained the urinary protein. It should be noted, however, that not from all normal urines peak Ia was obtained. Total nitrogen of the protein fraction amounted to 12.2 - 17.7 mg. N/24 hr.

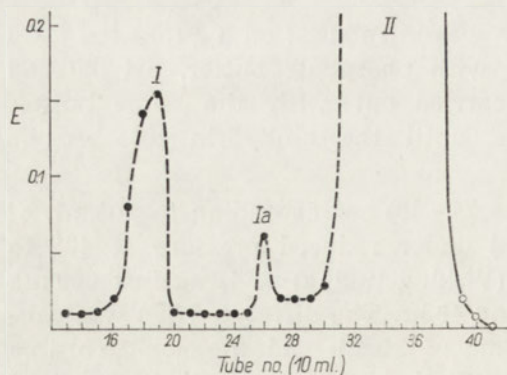


Fig. 1. Gel filtration of normal urine on Sephadex G-75 column equilibrated and eluted with phosphate buffer, pH 6.0; I, 0.10. (—), $E_{570\text{ m}\mu}$ (ninhydrin test); (---), $E_{280\text{ m}\mu}$

and constituted 0.15 - 0.20% of the total urinary nitrogen. Nitrogen of the protein peaks corresponded to daily excretion of 75 - 110 mg. protein (calculated using the coefficient 6.25). The α -amino nitrogen of the separated protein, which was determined in two cases, constituted 1.5 and 2.4% and after hydrolysis 11.6 and 11.2% of the total protein nitrogen.

The protein-free peak *II* (tubes no. 27 - 40) contained 85 - 93% of the total urinary nitrogen. The remaining low molecular nitrogen compounds of the urine, present in subsequent fractions and not reacting with ninhydrin (but showing absorption at 280 $m\mu$), were not examined.

The non-diffusible material (*FN*) of peak *II* contained 3.2 - 4.4% of the total urinary nitrogen. The α -amino nitrogen, determined in two urine samples, represented 2.2 and 4.2% and after hydrolysis 20 and 25% of the total nitrogen of the *FN* fraction. The separation of this fraction on a Sephadex G-25 column gave 4 peaks (Fig. 2a). The first three corresponded to peptides with a high sugar content and the fourth one

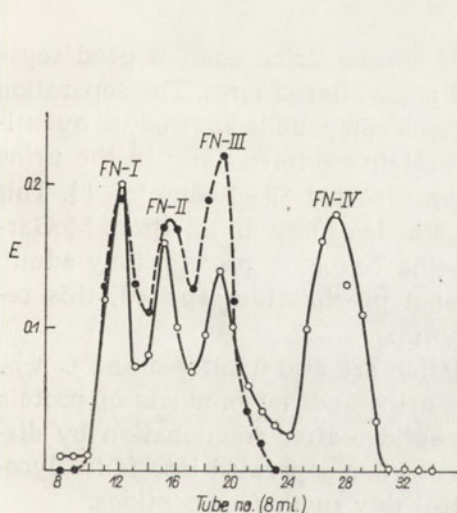


Fig. 2a

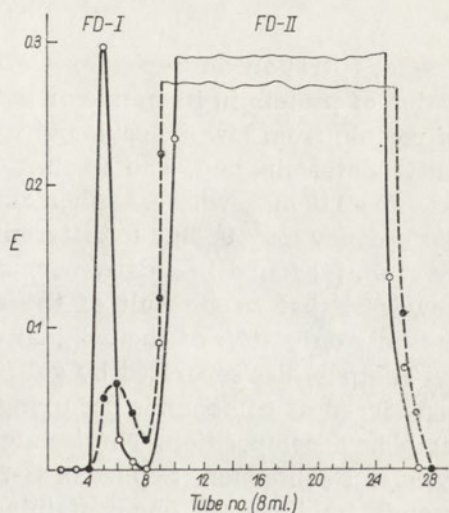


Fig. 2b

Fig. 2. Sephadex G-25 gel filtration of (a), non-diffusible material and (b), diffusible material from peak *II* (Fig. 1). Water was used as eluent. (—), $E_{570\text{ m}\mu}$ (ninhydrin test); (---), $E_{620\text{ m}\mu}$ (anthrone test).

contained sugar-free compounds. It should be mentioned that in some cases additional ninhydrin-positive peaks were observed between *FN* peaks *II* and *III* and between *III* and *IV*.

The diffusate (*FD*) separated on Sephadex G-25 gave two fractions (Fig. 2b). According to the accepted criteria of filtration on dextran gel, peak *FD-I* corresponded to high, and peak *FD-II* to low molecular nitro-

gen compounds. Peak *FD-I* contained about 1 mg. of total nitrogen and the α -amino nitrogen represented 18% of this amount.

Paper chromatography of fractions from peaks *FN I-IV* and *FD-I* revealed their heterogeneity, most marked for fraction *FN-III* both with respect to ninhydrin-positive and sugar components. From this fraction five ninhydrin-positive bands were obtained which also gave with aniline-phthalate a reaction of greater intensity than that given by any other separated peptide. Peak *FN-II* contained at least two peptides; in the slower migrating one sugars predominated over amino acids. Two peptides linked with sugars were also found in peak *FN-I*. The fraction of peak *FN-IV* represented at least three substances but contained no sugar components. Peak *FD-I* separated into three peptides; one of them, that giving the strongest reaction with ninhydrin, possessed a slight content of sugar. The glycopeptide character of the isolated compounds was confirmed by the analysis of the amino acid and sugar composition of their hydrolysates.

DISCUSSION

Gel filtration on Sephadex G-75 of normal urine enables good separation of protein in its native or but slightly altered form. The separation of protein from low molecular nitrogenous compounds allowed its quantitative determination. The amount of protein excreted daily in the urine was 75 - 110 mg., whereas other authors reported 39 - 70 mg. [cf. 8]. This discrepancy may be due to different methods of protein isolation. McGarry *et al.* [6] found in a daily normal urine 70 mg. of protein, they admit, however, that as a result of the alcohol precipitation applied, this represents only 70% of the original content.

The peptides separated by gel filtration are also unaltered and can be considered as compounds occurring in urine and not products of protein simplex decomposition. The isolated peptides after fractionation by dialysis and subsequent Sephadex G-25 filtration separated into four glycoprotein fractions and one containing probably sugar-free peptides.

The occurrence in urine of sugar-bound peptides has been already reported by some authors [1, 11]. Compounds of this type were isolated from the urine by Bourrillon *et al.* [2] from the non-diffusible fraction soluble in 50% ethanol. They emphasized, however, that the treatment with alcohol lead to formation of dialysable compounds, i.e. to partial degradation of the material. Lundblad & Berggård [5] separated on Sephadex G-25 (fine) low molecular sugar compounds of normal urine. The elution patterns of the diffusible material obtained by these authors differed from ours, more sugar-containing fractions being present in their material. This discrepancy may be due to the greater amount of urine analysed, and smaller particle size of the Sephadex used by Lundblad & Berggård.

In view of the presence of neutral sugars and amino sugars in the protein fraction and in the peptide fractions *FD* and *FN*, the increment in α -amino nitrogen after hydrolysis does not allow any conclusions to be made as to the length of the peptide chain. However, considering the differences in the magnitude of the protein, and *FN* and *FD* peptide molecules after separation of Sephadex G-75 and the participation of α -amino nitrogen after hydrolysis in the total nitrogen of these fractions, it may be concluded that the urinary proteins possess a higher content of sugar components than the peptide compounds.

During filtration of the diffusible peptide (*FD*) there appeared fractions almost completely excluded from the gel, i.e. apparently possessing higher molecular weight than the compounds contained in the non-diffusible peptide (*FN*). This may be explained by the fact, already observed by other authors, that separation of substances on Sephadex is not dependent only on the action of the gel as a molecular sieve.

SUMMARY

By Sephadex G-75 gel filtration, proteins and peptides from normal urine were isolated. The amount of daily protein excretion was 75 - 110 mg. The peptide peak gave on further fractionation 4 non-homogenous glycopeptide fractions and one fraction containing sugar-free peptides.

REFERENCES

- [1] Bode F., Becker G. & Böhle E. - *Naturwiss.* **40**, 274, 1953.
- [2] Bourrillon R., Cornillot P. & Got R. - *Clin. Chim. Acta* **7**, 506, 1962.
- [3] Fister H. J., *Manual of standardized procedures for spectrophotometric chemistry*. N-16 a.l. Standard Scientific Supply Corp., New York 1950.
- [4] Kos S., *I Krajowy Kongres Biochemii, Łódź 1963*. Abstr. Comm. p. 67.
- [5] Lundblad A. & Berggård I. - *Biochim. Biophys. Acta* **57**, 129, 1962.
- [6] McGarry E., Sehon A. H. & Rose B. - *J. Clin. Invest.* **34**, 832, 1955.
- [7] Noworytko J. & Sarnecka-Keller M. - *Acta Biochim. Polon.* **2**, 91, 1955.
- [8] Owen J. A. - *Advanc. Clin. Chem.* **1**, 238, 1958.
- [9] Partridge S. M. - *Nature* **164**, 443, 1949.
- [10] Rosen H. - *Arch. Biochem. Biophys.* **67**, 10, 1957.
- [11] Sarnecka-Keller M. - *Acta Biochim. Polon.* **8**, 389, 1961.
- [12] Sarnecka-Keller M. - *Bull. Acad. Polon. Sci., Ser. Biol.* **11**, 9, 1963.
- [13] Skarżyński B. & Sarnecka-Keller M. - *Advanc. Clin. Chem.* **5**, 107, 1962.
- [14] Stein W. H. - *J. Biol. Chem.* **201**, 45, 1953.
- [15] Sugar E. A. - *Clin. Chim. Acta* **8**, 347, 1963.

IZOLOWANIE I WSTĘPNY ROZDZIAŁ PEPTYDÓW MOCZU PRZY ZASTOSOWANIU FILTRACJI PRZESZ ŻELE

Streszczenie

Zastosowanie frakcjonowania na żelu Sephadex G-75 pozwala na wydzielenie białek i peptydów moczu. Normalny moczu dobowy zawiera 75 - 110 mg. białka. Dializa i dalsze frakcjonowanie wyosobnionych peptydów pozwoliły na wyodrębnienie 4 niejednorodnych frakcji glikopeptydowych oraz jednej zawierającej peptydy wolne od komponentów cukrowych.

Received 20 December 1963.

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ARYLSULPHATASE FROM PIG INTESTINAL MUCOSA

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It has been established recently, on the basis of the works of Roy [11, 12] and Dodgson *et al.* [4] that three different arylsulphatases (arylsulphate sulphohydrolase, EC 3.1.6.1) are present in the mammalian tissues. Two of these enzymes, i.e. arylsulphatases A and B are readily soluble, whereas arylsulphatase C is firmly bound to the cellular structures. The soluble and insoluble sulphatases differ with respect to hydrolysis of two typical substrates. Sulphatases A and B hydrolyse more rapidly nitrocatechol sulphate and the insoluble sulphatase C, *p*-nitrophenyl sulphate.

Whereas the presence of both soluble arylsulphatases in the intestinal mucosa has been observed earlier [9, 5], no attempt at their purification from this tissue has been undertaken. In the course of this study, soluble arylsulphatase from pig intestinal mucosa has been partly purified and some properties of the enzyme have been investigated.

MATERIALS AND METHODS

Abbreviations used: NCS, nitrocatechol sulphate; NPS, *p*-nitrophenyl sulphate; PCMB, *p*-chloromercuribenzoic acid.

Substrates and inhibitors. Dipotassium nitrocatechol sulphate (2-hydroxy-5-nitrophenyl sulphate), was prepared according to the method of Roy [14]. *p*-Nitrophenyl sulphate was synthesized by the Burkhardt & Lapworth method [2], as described by Dodgson & Spencer [3]. PCMB and *N*-ethylmaleimide were obtained from L. Light, Colnbrook, Bucks, England. Iodine and other reagents were of analytical purity.

Arylsulphatase assay. Arylsulphatase activity was determined essentially by the method of Roy [10]. According to this, 0.6 ml. of the enzyme solution was incubated with 0.4 ml. of the substrate (5 mM, in 0.5 M-acetate buffer, pH 5.6) at 37°. After an appropriate time of incubation, 0.5 ml. of 1 N HCl was added, followed by 3.5 ml. of alkaline quinol reagent. The colour of nitrocatecholate ion was measured in Coleman-

-Junior spectrophotometer at 520 m μ . When NPS was used as a substrate, the reaction was stopped by addition of 4 ml. of 0.2 N-NaOH and the yellow colour of the liberated *p*-nitrophenol was measured at 430 m μ . The amount of the enzyme which hydrolyses 1 μ mole of the substrate per minute at 37° under the conditions of assay, was taken as a unit of arylsulphatase activity.

Protein concentration was determined with the Folin & Ciocalteu phenol reagent [6].

Enzyme preparation. Acetone powder prepared from pig intestinal mucosa according to Brady [1] was used as a starting material for the purification procedure. The powder stored *in vacuo* above P₂O₅ at -10° was active for many weeks.

The extraction and the initial steps of the purification were carried out by a procedure similar to that used by Roy for the purification of arylsulphatase A from ox liver [11]. Extraction was carried out with 0.2 M-acetate buffer, pH 5.6, 2 mM with respect to EDTA. The same buffer was used also in other steps of the procedure, unless otherwise stated. The suspension of acetone powder in the buffer (100 ml. per 10 g. of the powder) was autolysed for 1 hr. at 37°. After centrifugation at 0° the residue was resuspended in the next portion of the buffer, left overnight at 2° and centrifuged. The combined supernatants were clarified by centrifugation for 20 min. at 10 000 r.p.m. This extract was fractionated further with acetone at -8°, the precipitate obtained between 42 and 60% of acetone was collected and dissolved in the buffer. From this solution the enzyme was precipitated by 0.3 ammonium sulphate saturation. The precipitate was centrifuged and dissolved in a small volume of the buffer and the precipitation with ammonium sulphate repeated. The fraction precipitated by 0.15 ammonium sulphate saturation was separated and extracted with buffer. This extract was combined with the next fraction precipitated between 0.15 and 0.4 ammonium sulphate saturation, and the obtained solution desalted on the Sephadex G-25 column, filled with 5 mM-tris-maleate buffer, pH 7.2. The desalted solution was then applied on the DEAE-cellulose column (1 \times 12 cm.) and 100 ml. of 5 mM-tris-maleate buffer, pH 7.2, was run through the column. The washing with this buffer removed some inactive protein. The enzyme was then eluted with 0.2 M-acetate buffer, pH 5.6. The active fractions were pooled and the enzyme was adsorbed on alumina C γ gel prepared according to Keilin & Hartree [7]. After separation by centrifugation, the gel was washed with water and the enzyme eluted with a small volume of 0.5 M-acetate buffer, pH 5.6, containing 2% of ammonium sulphate. The obtained solution was finally desalted on the column of Sephadex G-25 and stored at -10° without loss of activity. This preparation was used for all experiments aimed at the characterization of the enzyme.

RESULTS

In effect of the applied purification procedure, arylsulphatase was purified about 150 times in comparison with the initial extract of acetone powder. The purification achieved in the individual steps of the procedure is given in Table 1.

Table 1

Purification of arylsulphatase

The enzyme unit is defined as the amount of enzyme which hydrolyses 1 μ mole of nitro catechol sulphate per 1 min. under the conditions described in the text. The aliquots of the crude extract and other solutions, with exception of step 5, were desalted prior to the activity determinations.

Stage	Total activity (units)	Specific activity (units/mg. protein)
1. Extract of acetone powder	7400	0.12
2. Acetone fraction, 42 - 60%	4550	0.50
3. 1st ammonium sulphate precipitation	3700	2.44
4. 2nd ammonium sulphate precipitation	2960	3.80
5. DEAE-cellulose	940	5.90
6. Alumina C γ gel eluate	560	18.65

The purified preparation hydrolysed actively NCS, showing only negligible activity toward NPS. At pH 5.6 (acetate buffer) the ratio of activities (NPS:NCS) was about 1:1000; the ratio increased to 1:500 when the activity toward NPS was measured at pH 7.2 (tris - maleate buffer).

Fig. 1 presents the effect of pH on the activity of the purified arylsulphatase preparation. Apparently the value of pH optimum varied in relation to the kind of the buffer. In acetate buffer the maximum of activity was at pH 5.4, in tris - maleate buffer it was shifted to about pH 5.7, the enzyme activity being lower in tris - maleate buffer.

The effect of substrate concentration was studied with NCS used in the concentration range from 0.5 mM to 30 mM (Fig. 2). No inhibition by the excess of the substrate was observed. The value of Michaelis constant calculated from the Lineweaver-Burk plot was for this substrate 0.7 mM.

Arylsulphatase was inhibited by many anions, among which phosphate and sulphite acted in the lowest concentrations. The enzyme was also sensitive to the action of some thiol reagents. Iodine and PCMB

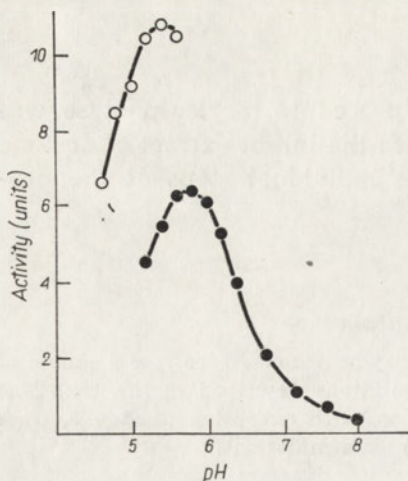


Fig. 1

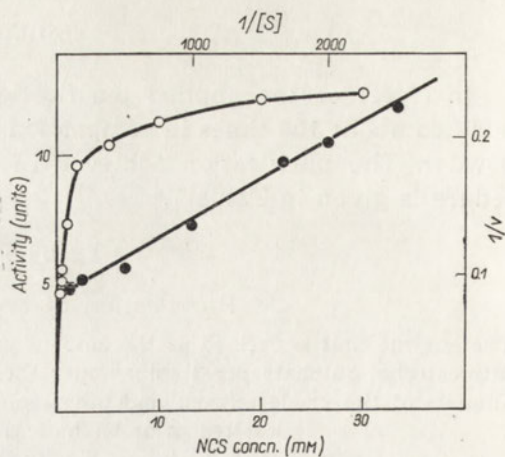


Fig. 2

Fig. 1. Effect of pH on arylsulphatase activity. The incubation mixture contained the enzyme, buffer of the appropriate pH and 5 mM-nitro catechol sulphate. (O), 0.1 M-Acetate (mean values from six series of determinations); (●), 0.1 M-tris - maleate (mean values from three series of determinations).

Fig. 2. Effect of nitro catechol sulphate concentration on arylsulphatase activity. The incubation mixture contained the enzyme, NCS and 0.2 M-acetate buffer, pH 5.6, in the total volume of 5 ml. Duplicate 0.5 ml. portions were withdrawn in different time intervals for the measurement of substrate decomposition and the initial activity was calculated from the obtained results. The Lineweaver-Burk plot constructed with the values taken from the main curve is drawn on the same diagram. The values are the means of two series of experiments.

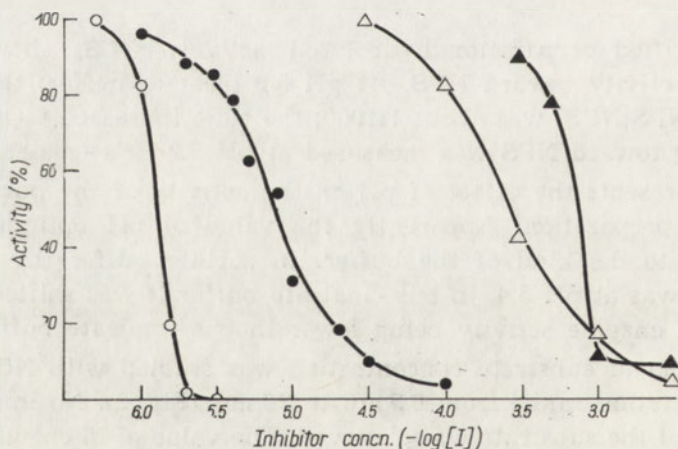


Fig. 3. Effect of inhibitor concentration on arylsulphatase activity. The enzyme was preincubated with inhibitor for 15 min. at 37° and then the activity was measured with 5 mM-NCS in acetate buffer, pH 5.6. (O), Iodine, (●), PCMB; (Δ), KH_2PO_4 ; (▲), Na_2SO_3 .

Table 2

Effect of inhibitors on arylsulphatase activity

The enzyme was preincubated with inhibitor for 15 min. at 37°. Conditions of assay: nitrocatechol sulphate 5 mM, acetate buffer 0.2M, pH 5.6. pI_{50} value represents the negative logarithm of inhibitor concentration producing 50% inhibition. The values of pI_{50} were obtained from the data of Fig. 3.

Inhibitor	Inhibitor concentration	Inhibition (%)	pI_{50}
KH_2PO_4	0.03 mM - 3 mM	—	3.6
Na_2SO_3	0.3 mM - 3 mM	—	3.2
NaF	10 mM	64.2	—
$Na_2S_2O_3$	10 mM	53.5	—
$Na_4P_2O_7$	10 mM	48.1	—
NaCN	10 mM	10.1	—
NaCN	20 mM	32.0	—
$NaAsO_2$	10 mM	18.6	—
Na_2SO_4	10 mM	6.3	—
Na_2SO_4	0.1 M	55.6	—
NaCl	20 mM	0.0	—
I_2	0.5 μ M - 3 μ M	—	5.9
PCMB	1 μ M - 0.1 mM	—	5.1
N-Ethylmaleimide	1 mM	0.0	—

caused a marked inhibition in the concentrations as low as 2 μ M and 10 μ M, respectively (Fig. 3). No inhibition was, however, caused by N-ethylmaleimide, known as a very potent thiol reagent. Data concerning the inhibitory effect of salts and thiol reagents are cumulated in Table 2.

DISCUSSION

The soluble arylsulphatase isolated from pig intestinal mucosa can be identified as arylsulphatase A. The enzyme was precipitated from a crude extract between 42 and 60% of acetone concentration, similarly as Roy's arylsulphatase A from ox liver [11]. The isolated enzyme resembles the purified arylsulphatase A from ox liver in several properties. The dependence on the concentration of NCS, the K_m value for this substrate and the general pattern of inhibition by anions are similar to the analogous properties of the liver enzyme. Nevertheless, some properties of the studied enzyme differ from those reported for arylsulphatase A obtained from other sources. The ratio of activities toward NPS and NCS, for example, is definitely lower than any value reported [13] for either arylsulphatase A or arylsulphatase B. Also the relatively low inhibitory effect of sulphate ions is not typical for arylsulphatase A.

The degree of purification of the studied enzyme is relatively low in comparison with that obtained by Roy. It should be emphasized,

however, that the aliquots of the initial extract were desalted prior to the activity determinations, thus ensuring the removal of inhibitory ions which are normally present. If the determinations were carried out without such precautions, the activity of the initial acetone powder extract would have been much lower due to the inhibition by phosphate ions present and the purification factor would be several times greater.

It is interesting that the studied arylsulphatase is strongly inhibited by iodine and PCMB, which indicates that the thiol group may be important for the activity of the enzyme. The lack of the inhibitory effect of *N*-ethylmaleimide suggests that the thiol groups in the activity site or in its proximity are not accessible to all thiol reagents, or that the attachment of *N*-ethylmaleimide does not interfere with the enzymic activity. The inhibitory effect of thiol reagents has been also observed recently by Wortman [15] on the chromatographically separated fractions of corneal extracts exhibiting arylsulphatase activity. The activity was strongly inhibited by *p*-hydroxymercuribenzoate, but *N*-ethylmaleimide and other thiol reagents were without effect.

Despite of the relatively wide occurrence of arylsulphatases in different mammalian tissues, no definite physiological function can be ascribed to these enzymes. It can hardly be assumed that any digestive role may be played by these enzymes, despite of the fact that arylsulphatase activity is present in intestinal mucosa and has also been detected in intestinal juice [8]. Neither there is any indication for participation of intestinal arylsulphatase in the known physiological functions of this tissue.

SUMMARY

Soluble arylsulphatase from pig intestinal mucosa has been purified and some properties investigated. The enzyme hydrolyses rapidly nitrocatechol sulphate, showing only negligible activity toward *p*-nitrophenyl sulphate. With nitrocatechol sulphate the optimum pH is 5.4 and the Michaelis constant 0.7 mM. The enzyme is inhibited by many anions, especially by phosphate and sulphite ions, and by some thiol reagents. The enzyme can be classified as arylsulphatase A.

REFERENCES

- [1] Brady T. - *Biochem. J.* **36**, 478, 1942.
- [2] Burkhardt G. N. & Lapworth A. - *J. Chem. Soc.* 684, 1926.
- [3] Dodgson K. S. & Spencer B., in *Methods of Biochemical Analysis* (D. Glick, ed.) vol. 4, p. 211, Interscience, New York & London 1957.
- [4] Dodgson K. S., Spencer B. & Thomas J. - *Biochem. J.* **59**, 29, 1955.
- [5] Dodgson K. S., Spencer B. & Wynn S. H. - *Biochem. J.* **62**, 500, 1956.
- [6] Folin O. & Ciocalteu V. - *J. Biol. Chem.* **73**, 627, 1927.

- [7] Keilin D. & Hartree E. F. - *Proc. Roy. Soc. London B* **124**, 397, 1938.
- [8] Oleksy J., Szafran H. & Szafran Z. - *Acta Biochim. Polon.* **8**, 43, 1961.
- [9] Robinson D., Smith J. N. & Williams R. T. - *Biochem. J.* **49**, LXXIV, 1951.
- [10] Roy A. B. - *Biochem. J.* **53**, 12, 1953.
- [11] Roy A. B. - *Biochem. J.* **55**, 653, 1953.
- [12] Roy A. B. - *Biochem. J.* **57**, 465, 1954.
- [13] Roy A. B. - *Biochem. J.* **64**, 651, 1956.
- [14] Roy A. B. - *Biochem. J.* **68**, 519, 1958.
- [15] Wortman B. - *Arch. Biochem. Biophys.* **97**, 70, 1962.

SULFATAZA ARYLOWA Z BŁONY ŚLIZOWEJ JELITA WIEPRZOWEGO

Streszczenie

Rozpuszczalną sulfatazę aryłową z błony śluzowej jelita wieprzowego oczyszczono i zbadano niektóre jej własności. Enzym hydrolizuje aktywnie siarczan nitrokatecholu i wykazuje tylko nieznaczną aktywność w stosunku do siarczanu *p*-nitrofenolu. Hydroliza siarczanu nitrokatecholu wykazuje maksimum przy pH 5.4, a stała Michaelisa dla tego substratu wynosi 0.7 mM. Enzym ulega zahamowaniu pod wpływem szeregu anionów, szczególnie jonów fosforanowych i siarczynowych i jest wrażliwy na działanie niektórych odczynników tiolowych. Na podstawie zbadanych własności enzym zaklasyfikowano jako sulfatazę aryłową A.

Received 20 December 1963.

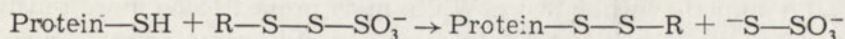
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RADIOPROTECTIVE PROPERTIES OF CYSTEINETHIOSULPHONATE

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In 1949 Patt *et al.* [9] observed that cysteine prevents X-irradiation damage in living organisms. In the course of further studies it was found that some other sulphur compounds, glutathione [3], cysteamine and cystamine [1], aminoethylisothiuronium [4] and 1-amino-2-mercapto-propane [16], possess similar protective properties. It is generally assumed that sulphur compounds protect against indirect effect of radiation by binding free radicals and by lowering the concentration of oxygen, and against direct effects by forming mixed bisulphides with proteins [2]. Protection against direct effects of radiation is thought to be of greater importance [7], however, it is not clear why cystamine has a protective effect while cystine [6] is inactive, whereas both cysteamine and cysteine are active.

In our experiments we employed the sodium salt of cysteinethiosulphonic acid, $\text{HOOC} \cdot \text{CHNH}_2 \cdot \text{CH}_2 \cdot \text{S} \cdot \text{S} \cdot \text{SO}_3\text{Na}$ (CTS) synthesized for the first time in 1958 [13, 14]. As may be seen from the formula, this compound may be regarded as a mixed bisulphide formed from cysteine and thiosulphate. It was shown [14] that the bisulphide linkage in CTS is much less stable than that in cystine, and therefore it could be anticipated that CTS would readily transfer the cysteine residue to protein thiol groups according to the reaction:



To study the possibility of formation of such compounds *in vivo*, CTS labelled with ^{35}S in the position directly adjacent to carbon was synthesized: $\text{HOOC} \cdot \text{CHNH}_2 \cdot \text{CH}_2 \cdot ^*\text{S} \cdot \text{S} \cdot \text{SO}_3\text{Na}$.

Taking into account the properties of CTS, a study was undertaken of its protective effect and its toxicity. If it could be shown that *in vivo* CTS forms mixed bisulphides and simultaneously protects against irra-

diation, some light would be thrown on the mechanism of the protective action of sulphur compounds, and an explanation may perhaps be obtained for the rather unexpected inactivity of cystine.

MATERIALS AND METHODS

Cysteinethiosulphonic acid was prepared by the method of Szczepkowski [13, 14] starting from L-cysteine (Fluka, Switzerland) and sodium thiosulphate (pure, Gliwice, Poland). Freshly prepared CTS was used for the experiments. The purity of the preparations was checked chromatographically, using the solvent system of *n*-butan-1-ol - acetone - water (2:2:1, by vol.). The spots were stained with isatin and with silver nitrate, with which CTS gives a brown silver sulphide. For the synthesis of radioactive CTS, DL-[³⁵S]cystine (Radiochemical Centre, Amersham, England) was used. Autoradiograms were made on rentgen films (Kodak, England).

Studies on the toxicity and protective effect of CTS were carried out on mice of the C57 (black) strain and on white mice of a local strain. The age of the animals ranged from 40 to 50 days and body weight from 15 to 25 g. CTS solutions of varying concentrations were injected intraperitoneally, the volume being always 0.1 ml. per 10 g. body weight.

Irradiation was administered from a rentgen apparatus (G. Massiot Co., France) at 120 kV and 15 mA, employing a 2 mm. Al filter. The time of irradiation, depending on dosage required and yield of the apparatus, was from 8.2 to 17.5 min. Whole body irradiation was applied, the distance of the mice from the source of radiation being 32 cm. To restrict the movements of the animals during irradiation, they were put into cardboard boxes of 13 cm. diameter divided into small compartments. Under these conditions it was possible to achieve whole body irradiation with a practically uniform dosis.

RESULTS

Toxicity of CTS. Experiments with C57 (black) mice showed that in series composed of 5 animals all of them survived the CTS dose of 2 g./kg. body weight while doses of 2.5 g./kg. or more caused the death of all the animals within 13 hr. White mice proved to be more sensitive, as 1.5 g./kg. was the highest safe dose, and only some animals survived the dose of 2 g./kg. Histological examination of the mice which died within 13 hr. after the injection consistently showed blood extravasations and capillary hyperemia. Focal edema of the lungs and brain, and necrosis of the kidneys with granular degeneration of the epithelial cells of the tubules were also observed. Similar but less pronounced changes were seen in the livers. These observations indicate that the toxic effect of CTS consists mainly in damaging the vascular walls,

especially of the capillaries, leading to altered permeability manifested by hyperemia, extravasations and edema. The lesions in the renal tubular epithelium and in the liver point to a harmful effect of CTS on the cells of these organs. Mice which received the maximum safe dose did not differ significantly from control animals with respect to body weight or composition of the white blood cells during 30 days' observation after the injection. Histological examination of animals killed after that time also failed to disclose any visceral lesions.

Formation of mixed bisulphides with proteins. Six mice of the C57 (black) strain weighing approx. 20 g. each were given intraperitoneal injections of 0.3 ml. of solution containing 3 mg. of [^{35}S]CTS, equivalent to approx. 60 μc . Pairs of animals were killed 0.5, 1 and 2 hr. after the injection and the blood was immediately secured from the heart and large blood vessels. Serum separated from the blood was submitted to paper electrophoresis in veronal buffer of pH 8.6 and ionic strength 0.05. Electrophorograms were exposed for 3 months to obtain autoradiograms (Fig. 1), and then stained to locate the protein fractions. On the autoradiograms representing the blood withdrawn 0.5 hr. after the injection

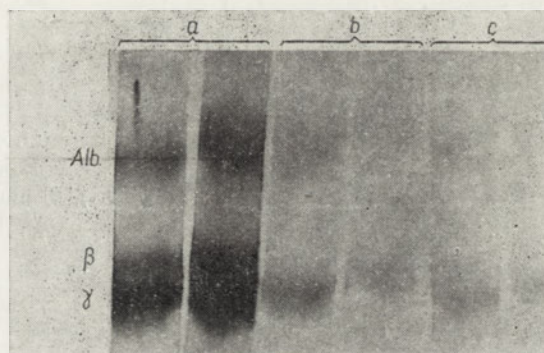


Fig. 1. Autoradiograms of electrophorograms of blood serum of mice which had been injected with CTS labelled with ^{35}S on sulphur of the cysteine moiety. Blood was taken (a), 0.5 hr.; (b), 1 hr.; and (c), 2 hr. after the injection.

of [^{35}S]CTS, darkening may be seen which corresponds to radioactivity bound with the γ - and β -globulin and albumin fractions. In view of CTS labelling it follows that the protein-bound radioactivity is derived from the cysteine residue containing ^{35}S , and it seems unlikely that whole CTS molecule bound ionically would persist after electrophoresis. The time of decline in protein-bound activity indicates that the cysteine residue is not incorporated into the protein molecule but remains bound, probably as cysteine-protein bisulphide.

For comparison, autoradiograms were made from the electrophorograms of the blood serum of mice injected with labelled cystine; they proved to be similar to those shown in Fig. 1, quantitative comparison, however, could not be made because of low solubility of cystine and impossibility of administering adequate doses.

Protecting action of CTS against irradiation. Two parameters were taken into account: time of CTS administration before irradiation and amount of the dose. Animals injected with 0.9% NaCl solution and subjected to identical doses of irradiation were used as controls. In Fig. 2 the survival time of mice after injection of 1 g. CTS/kg. at various intervals before and after irradiation with 700 r is presented. The most

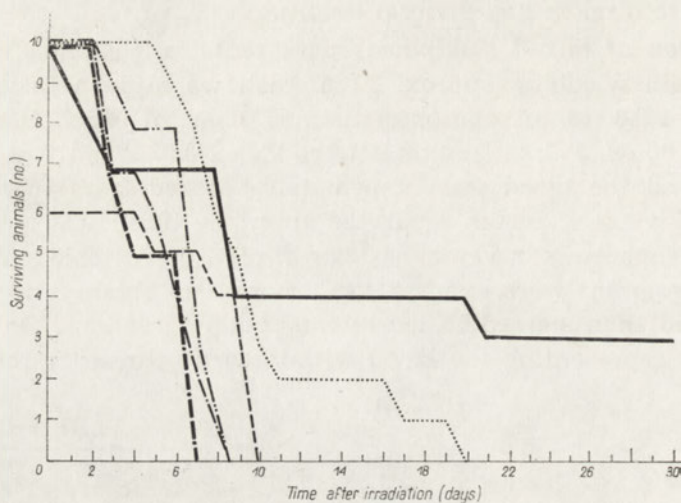


Fig. 2. Effect of the time of CTS administration on survival of white mice irradiated with 700 r. The animals were injected with 1 g. CTS/kg. body wt. (—), 5 min. after irradiation; and (— — —), 5 min., (...), 15 min., (-.-), 30 min., (-.-.-), 60 min. before irradiation. (.....), Controls, injected with 0.9% NaCl solution.

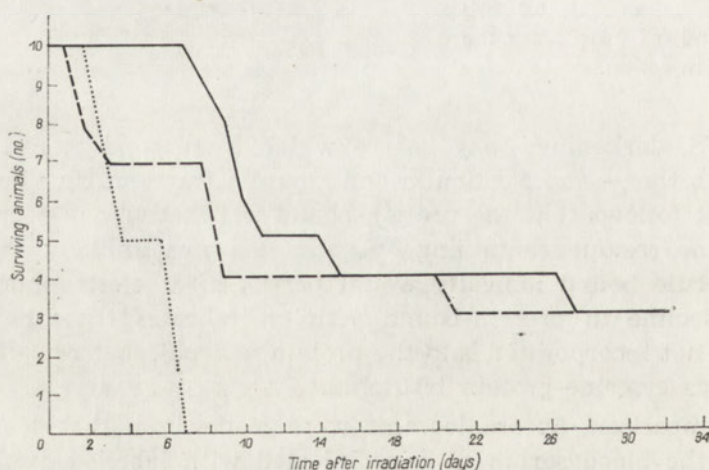


Fig. 3. Effect of CTS dose on survival of white mice irradiated with 700 r. CTS was injected 3-8 min. before irradiation. Dosis applied: (—), 0.25 g./kg. body wt.; (---), 1 g./kg. body wt.; (...), control.

pronounced protective effect of CTS was observed in the group of mice which received the injections 5 min. before irradiation. These animals survived longer than those of other groups, and 3 out of 10 survived for 6 months of observation. One female mouse in this group gave birth to a litter 38 days after irradiation, i.e. was fertilized in the course of the experiment. The animals which received injections of CTS 15 min. before irradiation also had a noticeably prolonged survival time, but after 20 days all of them were dead. The results for mice which received CTS 30 and 60 min. before irradiation were similar to those for the control group. In Fig. 3, the survival time for mice which received CTS in doses of 1 g./kg. and 0.25 g./kg. is presented. The results show that the protective action of CTS is not related to the dose applied.

DISCUSSION

Cysteinethiosulphonate, a product of the oxidation of cysteine and thiosulphate, is an asymmetrical organic-inorganic bisulphide. It can be regarded as a cysteine analogue but, in contrast to cystine, CTS is readily soluble in water. It was found previously [14] that the bisulphide linkage in CTS is less stable than in cystine. Further experiments confirmed these results; CTS was found to react rapidly with cysteine forming cystine and thiosulphate, and with glutathione forming cysteine-glutathione bisulphides (T. W. Szczepkowski, unpublished results). In the present work the transfer *in vivo* of [^{35}S]cysteine residue of CTS to protein thiol groups was demonstrated. It is particularly interesting that the radioactivity of cysteine was localized in the γ -globulin fraction which markedly decreases after irradiation [8].

The formation with protein of mixed bisulphide is supposed to be the main radioprotective mechanism of sulphur compounds [10, 11]. The protective effect of CTS found in the present experiments seems to support this assumption. It should be pointed out that the protective effect of CTS depends only on the cysteine component while thiosulphate [12], which may be split from CTS, and tetrathionate [5] have no protective properties. As a compound protecting against the effects of X-irradiation, CTS is characterized by a marked interval between the lethal and the protective dosis.

It seems possible, taking into account the presented results, to suggest a partial explanation why cystine has no protective effect. As Pihl & Eldjarn [11] have shown, cystine also forms mixed bisulphide compounds with proteins. In our experiments, the autoradiograms obtained after intraperitoneal injection of radioactive cystine were similar to those obtained after CTS injections. The difference in the effect produced by cystine and that of CTS is therefore probably

quantitative and may be due to the relative insolubility of cystine and its restricted penetration into the cell, although differences in the ability to form mixed bisulphides may also play a role.

SUMMARY

The toxicity of cysteinethiosulphonate (CTS), a water-soluble analogue of cystine, was determined and the protective effect of CTS against X-irradiation was observed. By injecting [^{35}S]CTS to mice it was shown that mixed bisulphides are formed *in vivo* between blood serum proteins and the cysteine moiety of CTS. Lack of protective effect of cystine is probably due to its low solubility.

REFERENCES

- [1] Bacq Z. M., Herve A., Lecomte J., Fisher F., Blevier J., Dechamps G., Le Bihan H. & Rayet P. - *Arch. Intern. Physiol. Biochem.* **59**, 442, 1951.
- [2] Bacq Z. M. & Alexander P., *Fundamentals of Radiobiology*. Pergamon Press, Oxford, 1961.
- [3] Chapman W. H., Sipe C. R., Eltzholz D. C., Cronkite E. P. & Chambers F. W. - *Radiology* **55**, 865, 1950.
- [4] Doherty D. C. & Burnett W. T., Jr. - *Proc. Soc. Exper. Biol. Med.* **89**, 312, 1955.
- [5] Langendorff H. & Koch R. - *Strahlentherapie* **95**, 535, 1954.
- [6] Langendorff H., Koch R. & Hagen Cl. - *Strahlentherapie* **100**, 137, 1956.
- [7] Lea D. A., *Action of Radiation on Living Cells*, Cambridge Univers. Press, London 1946.
- [8] Meynill G. & Mende de G. - *Bull. Soc. Chim. Biol.* **37**, 127, 1955.
- [9] Patt H. M., Tyree E. P., Straube R. L. & Smith D. E. - *Science* **110**, 213, 1949.
- [10] Pihl A., Eldjarn L. & Shapiro B., *Proc. 1st Intern. Congr. Peaceful Uses Atomic Energy, Geneva 1955*, **11**, 335, 1956.
- [11] Pihl A. & Eldjarn L., *Proc. 4th Intern. Congr. Biochemistry, Vienna 1958*, vol. 13, p. 43, Pergamon Press, London 1959.
- [12] Rozeronowa W. A., *Woprosy patogeneza eksperymentalnoj terapii i profilaktiki luczewoj bolezni*. p. 191, Medgiz, Moskwa 1960.
- [13] Szczepkowski T. W. - *Nature* **182**, 934, 1958.
- [14] Szczepkowski T. W. - *Roczniki Chemii* **35**, 571, 1961.
- [15] Tank L. I. - *Medicinska Radiologija*, **9**, 34, 1960.

WŁASNOŚCI CYSTEINOTIOSULFONIANU W ZAPOBIEGANIU USZKODZENIOM RADIACYJNYM

Streszczenie

Określono toksyczność i stwierdzono ochronie przed promieniowaniem X działanie cysteinotiosulfonianu (CTS), rozpuszczalnego analogu cystyny. Podając myszom [^{35}S]CTS wykazano, że *in vivo* powstają mieszane dwusiarczki między białkami surowicy krwi a cysteiną wchodzącą w skład CTS. Brak ochronnego działania cystyny można wytłumaczyć jedynie złą rozpuszczalnością tego związku.

Received 20 December 1963.

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NUCLEOLYTIC ENZYMES OF *THIOBACILLUS THIOPARUS*

II. RIBONUCLEASES AND THEIR PROPERTIES

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In our previous communications [6, 7] it was shown that crude extract of the cells of *Thiobacillus thioparus* completely degrades yeast RNA, liberating oligonucleotides, mononucleotides and nucleosides. By fractionating the extract with protamine, ammonium sulphate and by chromatography on CM-cellulose, DEAE-cellulose and Amberlite IRC-50, separation was achieved, and ribonucleases, nonspecific phosphodiesterase and 5'-nucleotidase were identified [5, 7]. In this study it is shown that the crude extract of the microorganism contains at least three ribonucleases differing with respect to optimum pH, rate of thermal denaturation and adsorptive properties, but probably endowed with the same specificity with regard to RNA.

MATERIAL AND METHODS

The method of harvesting *Th. thioparus* and of preparing the extracts was described previously [6]. Extracts of the ground cells prepared under mild conditions (0.1 M-phosphate - citrate buffer, pH 7.0), as well as those obtained with 0.25 N-H₂SO₄ at 0°, showed almost identical nucleolytic activity toward yeast RNA.

Yeast RNA (Merck, Darmstadt) was employed as the substrate for the estimation of RNase activity. Before use, the commercial preparation was purified by prolonged dialysis against water at 4°, then precipitated with two volumes of ethanol in the presence of 1% sodium acetate, washed with alcohol and ether, and finally dried *in vacuo*.

Beef pancreas RNase, three times crystallized, was obtained by the method of McDonald [3].

Column chromatography. Three types of ion exchangers were used in the purification procedure. CM-cellulose and DEAE-cellulose with 0.66 and 0.60 mEq/g. capacities were purchased from Serva Co. (Heidelberg, Germany); Amberlite IRC-50 (XE 64) 200 - 400 mesh was obtained

from L. Light & Co. (Colnbrook, England). The method of preparing the adsorbents was previously described [6].

Paper chromatography. Chromatography of the hydrolysis products of RNA was carried out with the following solvents: A, *n*-propanol-ammonia-water (55:10:35, by vol.) for separation of nucleotides [10]; B, 96% ethanol - 1 M-ammonium acetate (75:50, v/v), saturated with boric acid and added with conc. ammonia to pH 11, for separation of 3'-from 5'-phosphomononucleosides [8]; C, isopropanol - hydrochloric acid - water (65:35, v/v, and made up to 100 vol. with water) for separation of bases [13]. Two-dimensional separation was performed by means of high voltage electrophoresis in diluted formic acid at pH 2.35 [9] at 12 V/cm., 10-20 mA, during 6 hr. at 10°, and by paper chromatography in solvent A. In all cases Whatman no. 1 filter paper was used.

Enzyme assay. Activity of RNase was assayed as previously described [6] on the basis of increment of extinction at 260 m μ caused by soluble hydrolysis products of RNA.

RESULTS

Isolation of RNases from the cells of Th. thioparus. All the procedures involved in the preparation of the extract and in its further fractionation were carried out in the cold room (4°). The cell extract was first fractionated with ammonium sulphate, the fraction precipitated between 55 and 85% saturation being collected. After dissolving the precipitate in a small amount of water, the solution was dialysed against water and lyophilized. About 5 g. of the dry powder was then dissolved in 100 ml. of 0.01 M-sodium phosphate buffer, pH 6.0, and adsorbed on a column (3.5 \times 20 cm.) filled with CM-cellulose and equilibrated with the same buffer solution. After washing the column with about 800 ml. of the starting buffer, activity remaining in the column was eluted with convex gradient between 0.01 M and 0.2 M-sodium phosphate buffer, pH 7.5. The elution was followed by estimating protein at 280 m μ and determining RNase activity. Two protein peaks, designated I and II (Fig. 1), exhibiting high nucleolytic activity were obtained. Fraction I contained about 30% of the initial activity, and fraction II about 70%. Fractions corresponding to each peak were pooled and after dialysis against distilled water were lyophilized.

Fraction I was treated at 0° with sulphuric acid the concentration of which was adjusted to 0.25 N, and after 10 min. the solution was adjusted to pH 6.8. The precipitate was centrifuged off and discarded, and the solution was chromatographed on a column with Amberlite IRC-50 (3.7 \times 24 cm.) at pH 6.5 employing 0.2 M-sodium phosphate buffer. Under these conditions two separate peaks of RNase activity were obtained: IA and IB. The greater part of the protein and of the RNase

activity in the sample was eluted in peak *IA*, which emerged from the column after about 250 ml. of buffer solution had passed. Fraction *IA*, which contained about 60% of the activity applied to the column, was submitted to further purification on a column with DEAE-cellulose (2.1×19.9 cm.) with a convex gradient of NaCl at pH 6.8. The results

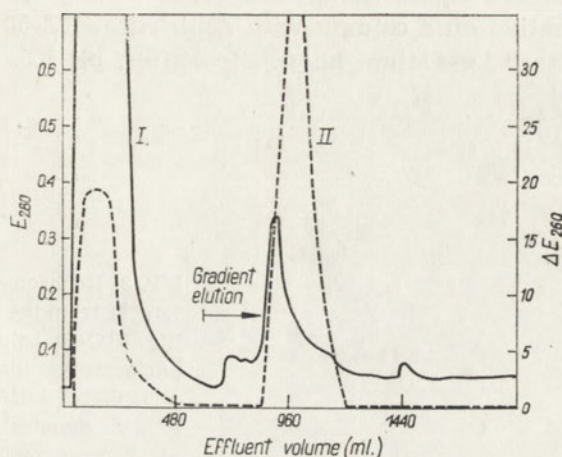


Fig. 1. Chromatography on CM-cellulose column of ammonium sulphate fraction. The mixing chamber with constant volume contained 500 ml. 0.01 M-sodium phosphate buffer, pH 6.0, to which 0.2 M-sodium phosphate buffer of pH 7.5 was added by drops. Fractions (12 ml.) were collected at the rate of 30 ml./hr. (—), Extinction at 280 mμ; (---), RNase activity.

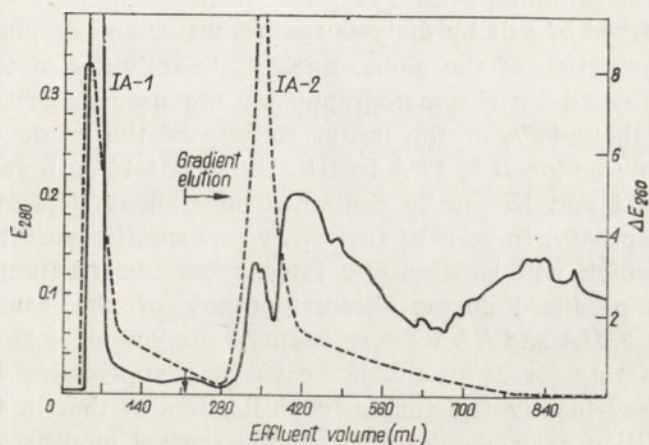


Fig. 2. Rechromatography of fraction *IA* (eluted from IRC-50 column, see text) on DEAE-cellulose column. The sample was adsorbed on the column equilibrated with 0.005 M-sodium phosphate buffer, pH 6.8, and after washing the column elution was performed with the convex gradient. To the mixing chamber of the constant volume, containing 200 ml. 0.005 M-sodium phosphate buffer, pH 6.8, 0.2 M- NaH_2PO_4 - 0.3 M-NaCl solution was introduced. Fractions (3 ml.) were collected at the rate of 90 ml./hr. (—), Extinction at 280 mμ; (---), RNase activity

and conditions of the separation are presented in Fig. 2. The RNase activity designated as the fraction *IA-2* was eluted at the beginning of the applied gradient, whereas most of the protein was eluted in the subsequent fractions. The corresponding active fractions were pooled, dialysed against water and lyophilized.

Peak *II* from the separation on CM-cellulose (Fig. 1) was submitted to further separation on a column with Amberlite IRC-50 (2.1×20 cm.), being eluted with 0.2 M-sodium phosphate buffer, pH 6.5. Fig. 3 presents

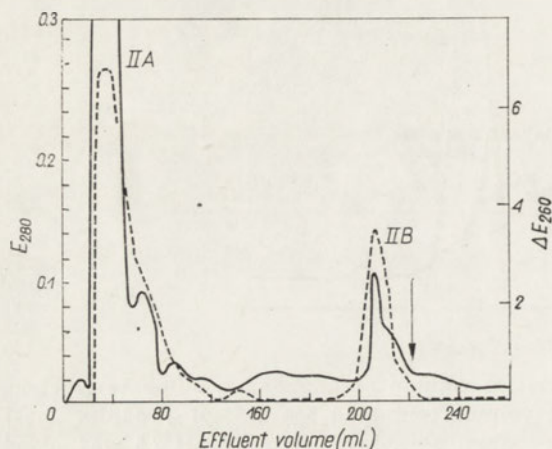


Fig. 3. Rechromatography of fraction *II* from the Fig. 1 on Amberlite IRC-50 column; elution was performed with 0.2 M-sodium phosphate buffer, pH 6.5; the arrow denotes the change to pH 7. Fractions (4 ml.) were collected at the rate of 16 ml./hr. (—), Extinction at 280 mμ; (---), RNase activity.

the resulting two fractions of RNase activity, *IIA* and *IIB*, and the corresponding protein peaks. Fractions from each peak were pooled separately, freed of salt by dialysis against water and lyophilized.

After calculation of the global nucleolytic activities of the different fractions obtained by chromatography on the used adsorbents, it was found that about 60% of the initial activity of the crude extract was recovered in fraction *IIA*, 17% in *IIB*, and about 10% in fraction *IA-2*. Fractions *IA-1* and *IB* jointly contained only about 15% of the initial nucleolytic activity. In view of their very low specific activity and other properties which will be discussed later, these two fractions appear to be artefacts produced during chromatography of the crude material. Enzymes *IA-2*, *IIA* and *IIB* were resistant to the low pH; 0.25 N-sulphuric acid at 0° acting for 24 hr. did not cause any appreciable inactivation. Nor were they inactivated during lyophilization, so that in the dried or frozen condition they could be stored for several months without loss of enzymic activity. None of these enzymes digests DNA, bis-*p*-nitrophenylphosphate or splits off appreciable amounts of inorganic phosphate during hydrolysis of RNA. The specific activity of the enzymes ranges from 2.5 to 2.7 ΔE_{260} units per mg. protein, and in this stage of purification the enzymes were concentrated about 300 times as compared with the crude extract.

Relation of activity to pH. As stated in the previous communication [6], crude extract of *Th. thioparus* cells showed three pH optima of hydrolysis of yeast RNA: at pH 5.5, 7.0 and 8.5. It may be seen in Fig. 4 that RNase IA-2 is most active at acidic pH (5.0 - 5.5), while RNase IIB

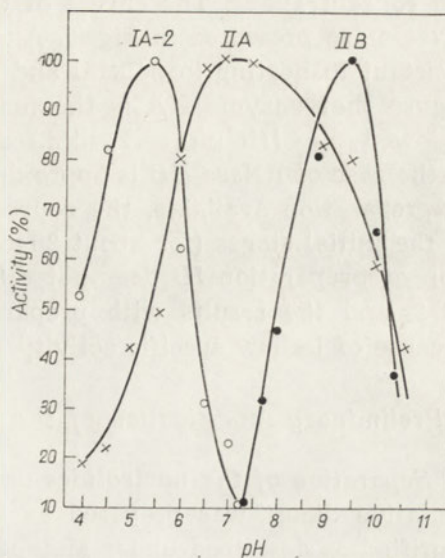


Fig. 4. Effect of pH on activity of *Th. thioparus* RNases. The reaction mixture contained: 0.4 ml. 0.2 M-buffer solution, 0.4 ml. 0.6% RNA sodium salt solution in water and 0.4 ml. enzyme solution with the mean activity of 2.5 ΔE_{260} units per sample and was incubated at 37° for 60 min. Buffer solutions used: acetate buffer for pH 4.0 - 5.5; sodium phosphate buffer for pH 6.0 - 8.0; tris - HCl for pH 8.0 - 9.0; glycine - NaOH for pH 9.0 - 10.5.

at alkaline pH (9.5); optimum hydrolysis of RNA by RNase IIA is at neutral pH (7.0).

Thermal inactivation of RNases IA-2, IIA and IIB. In order to determine the effect of heating on the activity of the different ribo-

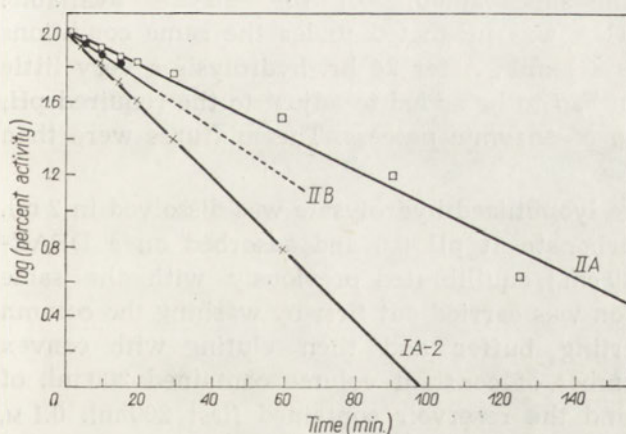


Fig. 5. Effect of heating at 100° on the activity of *Th. thioparus* RNases. Explanation in the text.

nucleases, enzyme solutions in 0.05 M-sodium phosphate buffer of pH 7.0 were heated at 100° in stoppered tubes. All the enzymes were first dialysed against the above buffer solution overnight, then insoluble protein was centrifuged off and discarded. Protein concentrations in the

different RNase samples were: *IA-2*, 1.1 mg./ml.; *IIA*, 1.2 mg./ml.; and *IIB*, 0.7 mg./ml. At intervals between 0 and 150 min., 0.5 ml. samples of the heated solutions were withdrawn and after immediate cooling to 0° the activity of the enzymes was estimated at their optimum hydrogen ion concentrations. The curves of thermal inactivation of the studied RNases are presented in Fig. 5. All the enzymes appear to be relatively resistant to heating in neutral and acid solutions. It will be seen in the Figure that enzyme *IIA* is the most thermostable one ($t_{1/2} = 31$ min.), followed by *IIB* ($t_{1/2} = 21$ min.) and *IA-2* ($t_{1/2} = 12$ min.). The result in the case of RNase *IIB* is approximate, as because of the small amount of preparation available, the course of denaturation was followed only in the initial stages (for about 20 min.). The course of thermal inactivation of preparation *IB* does not differ appreciably from that of RNase *IA-2*, and the results with preparation *IA-1* were not reproducible because of its low specific activity.

Preliminary investigation of the hydrolysis products of yeast RNA

Separation of the nucleotides on DEAE-cellulose at pH 8.6. A representative digest was prepared as follows: about 50 mg. of yeast RNA (purified as described under Methods) was dissolved in 10 ml. water and adjusted to the desired pH with NaOH solution. Next, to each sample of RNA solution the respective enzymes were added (*IA-2*, 2.3 mg.; *IIA*, 2.5 mg.; pancreatic RNase, 1 mg.), and the mixtures were incubated at 37° in a water bath. The solutions were adjusted occasionally to optimum pH by means of 0.1 N-NaOH. RNase *IIB* was not used in this experiment because of the small amount of the enzyme available. An identical sample of RNA was incubated under the same conditions without added enzyme as a blank. After 24 hr. hydrolysis a very little sodium hydroxide solution had to be added to adjust to the required pH, indicating the termination of enzymic process. The mixtures were then frozen and lyophilized.

A 25 mg. sample of the lyophilized hydrolysate was dissolved in 2 ml. of 0.01 M-ammonium bicarbonate at pH 8.6 and adsorbed on a DEAE-cellulose column (0.9 × 30 cm.) equilibrated previously with the same buffer solution [11]. Elution was carried out first by washing the column with 100 ml. of the starting buffer and then eluting with convex gradient; the mixing chamber of constant volume contained 200 ml. of 0.01 M-buffer of pH 8.6 and the reservoir contained first 200 ml. 0.1 M, then 200 ml. 0.2 M and finally 400 ml. 0.4 M-buffer of the same pH. All the chromatographic separations were performed under the same conditions and on the same column. Elution of nucleotides from the column was checked by measuring extinction at 260 mμ and 290 mμ. Fig. 6 depicts the results of separation of the hydrolysis products of

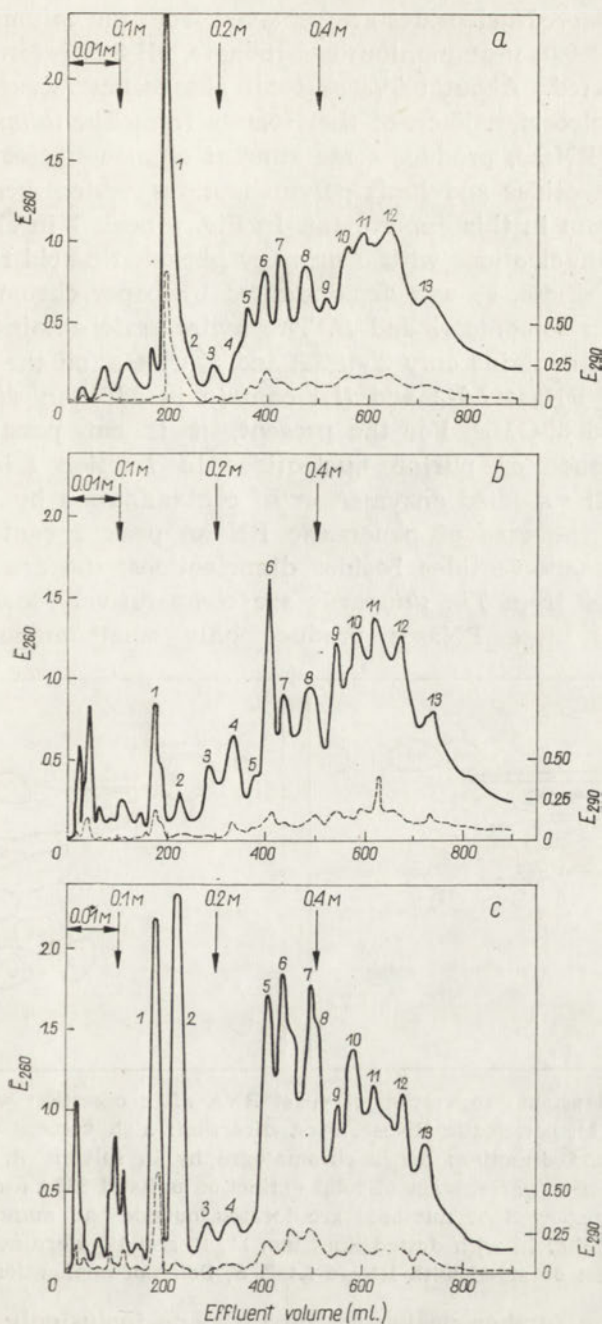


Fig. 6. Chromatogram of yeast RNA after digestion with RNases (a), IA-2; (b), IIA; and (c), pancreatic RNase, on a DEAE-cellulose column at pH 8.6. (—), Extinction at 260 mμ; (---), extinction at 290 mμ.

RNA obtained by the action of RNase IA-2, IIA and for comparison pancreatic RNase. Nucleosides are removed from the column by washing with 100 ml. of 0.01 M-ammonium bicarbonate, pH 8.6, before the gradient elution is started. About 0.5% of total absorbancy was found to be caused by nucleosides. Each of the RNases from *Th. thioparus*, as well as pancreatic RNase, produce some amount of mononucleotides, besides di- and trinucleotides and limit polynucleotides, which were not eluted from the column in this experiment. In Fig. 6 peak 1 in all three cases contains mononucleotides with a primary phosphoric acid residue at the carbon C₃ of ribose, as was demonstrated by paper chromatography of this fraction in solvents A and B. The hydrolysate obtained with pancreatic enzyme contains only 3'-UMP and 3'-CMP; and the hydrolysates obtained with RNase IA-2 and IIA contain in addition slight amounts of 3'-AMP and 3'-GMP. For the present, it is not possible to state whether presence of purine nucleotides in fraction 1 is a result of specificity of the studied enzymes, or of contamination by a nonspecific diesterase. In the case of pancreatic RNase, peak 2 contains a small amount of mononucleotides besides dinucleotides; the analogous peaks of the enzymes from *Th. thioparus* are comparatively low. It may be concluded that these RNases produce only small amounts of these

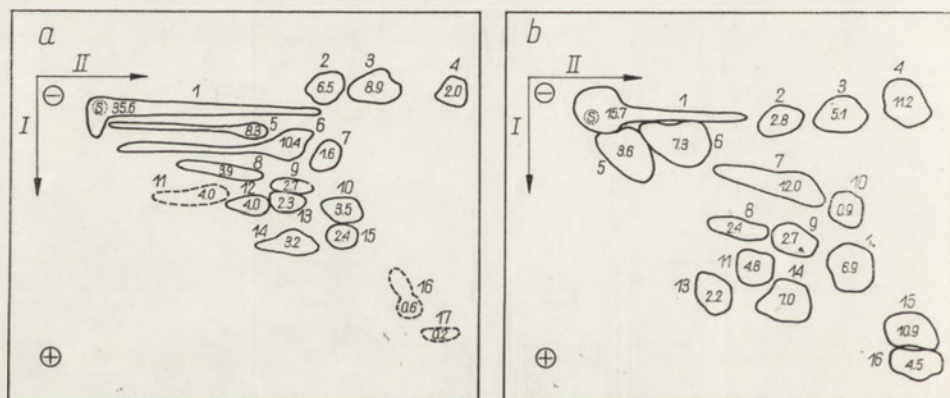


Fig. 7. Two-dimensional separation of yeast RNA after digestion with (a), RNase IA-2, IIA; and (b), pancreatic RNase. First direction: high voltage electrophoresis at pH 2.35; second direction: paper chromatography in solvent A. Values inside the spots are fraction percentages of total extinction units at 260 m μ of the material applied on the paper. Spot numbers are located outside the marked areas. The spots marked on Fig. 7a with dotted lines (no. 11, 16 and 17) were not found in the digest obtained with RNase IA-2. S, Point of application.

nucleotides. The further peaks, to the 13th one inclusively, differ with regard to height, but the essential profile of all three chromatograms is similar, indicating that the differences in the composition of the hydrolysates obtained with the tested enzymes are quantitative, but not qualitative in character. Slight differences in the profiles may also be due

to procedural variations during elution of the nucleotide material from the column. Further studies on the sequence of the different oligonucleotides should elucidate the problem.

Two-dimensional separation of the digest of RNA. Samples of hydrolysates prepared as described above were applied to Whatman no. 1 filter paper (0.1 ml. containing 2 mg. of lyophilized hydrolysate) and subjected first to electrophoresis in dilute formic acid at pH 2.35 [9], and then after drying, to descending chromatography in solvent A. During electrophoresis all the products moved toward the anode. After developing the chromatograms, contact prints were obtained from the maps under UV light. Spots showing absorption of ultraviolet light were cut out and eluted with water (18 hr., 30°), and their extinction was then determined at 250, 260, 275 and 290 m μ at pH 7.0. Fig. 7 shows the result of the separations. The values inscribed in the spots denote percentage of extinction at 260 m μ in relation to total extinction of the sample applied to the filter paper. The number of spots obtained under these conditions with different enzymes was 14-17. The ratios of extinction 250/260, 275/260 and 290/260 at pH 7.0, calculated for comparison for some of the corresponding spots, are given in Table 1. These

Table 1

The characteristics of some spots of two-dimensional separation of yeast RNA hydrolysates obtained after digestion with IA-2, IIA, and pancreatic RNases

The ratios of extinction: 250/260, 275/260, 290/260 after elution of the spots with water (18°, 24 hr.) are given.

RNase...	IA-2	IIA	Pancreatic
Spot 2			
250/260	0.86	0.80	0.88
275/260	0.59	0.62	0.58
290/260	0.11	0.13	0.14
Spot 3			
250/260	0.94	0.81	0.82
275/260	0.62	0.78	0.62
290/260	0.22	0.19	0.16
Spot 4			
250/260	0.85	0.82	0.87
275/260	—	1.04	1.01
290/260	—	0.34	0.27
Spot 14			
250/260	0.84	0.91	0.97
275/260	0.62	0.68	0.67
290/260	0.16	0.18	0.24

preliminary results show a very similar distribution of the spots on the paper and similar values of the extinction coefficients. They therefore provide further evidence that the enzymic action of both RNases IA-2 and IIA is very similar to that of pancreatic RNase.

Table 2

Limit polynucleotides remaining after hydrolysis with IA-2, IIA and pancreatic RNases

Percentages of remaining "core" represent the part of the total extinction at 260 mμ of the hydrolysates which remained at the point of application. Molar percentages are obtained after hydrolysis with perchloric acid and spectrophotometric estimation of the bases. G, guanine; A, adenine; C, cytosine; U, uracil. Pu/Py denotes molar ratio G+A/C+U. For comparison similar data for yeast RNA are included.

Ribonuclease	Core (%)	Molar proportion				Pu/Py
		G	A	C	U	
IA-2	46	48	31	11	10	3.8
IIA	35	62	23	8	7	6.0
Pancreatic	26	44	41	9	6	5.7
Yeast RNA	—	28	26	21	26	1.1

It may be seen in Fig. 7 that some part of the material placed on the filter paper remains at the origin in the form of tails during chromatography, regardless of the time of hydrolysis and of the amount of enzyme units added to RNA. By eluting these spots and determining their extinction (spots no. 1), it was found that about 40% of the total extinction values applied remained at the origin of the map. Analysis of the composition of the bases of these spots after hydrolysis with 60% HClO₄ at 100° for 1 hr. and after chromatography in solvent C gave the results which are presented in Table 2. It will be seen in the Table that RNases from *Th. thioparus* as well as pancreatic RNase markedly increase the ratio of purine/pyrimidine bases in the limit polynucleotides. An especially high content of guanylic nucleotide may be seen in the "core" remaining after RNase IIA. The remaining core can also be isolated by acidifying the hydrolysates or by dialysing off lower oligonucleotides.

DISCUSSION

From these studies and others (to be published) it can be seen that RNases from *Th. thioparus* can be classified as endonucleases which hydrolyse RNA with formation of small amounts of mononucleotides, lower oligonucleotides and core with 3'-phosphomonoester end groups. The preliminary results indicate that the enzymes preferentially hydrolyse bonds between pyrimidine nucleotides, and between pyrimidine

and purine nucleotides, as may be seen from the analysis of the soluble hydrolysis products of RNA and from the base composition of the limit polynucleotides.

The results also show that RNases *IA-2*, *IIA* and *IIB* can be distinguished one from another only on the basis of different abilities of adsorption on various adsorbents, optimum pH for hydrolysis of RNA and differences in the rate of thermal inactivation. This, of course, is not final evidence that they represent enzymes with different physico-chemical properties, and further investigation of this problem is needed. All the enzymes are resistant to elevated temperature and high hydrogen ion concentration.

The similarity of the enzymes from *Th. thioparus* to the pancreatic RNase is striking, both with regard to physico-chemical properties (thermostability, resistance to low pH, adsorption and optimum pH, especially of RNase *IIA*), and with regard to mode of action on RNA [1, 2]. In Figs. 6 and 7 it can be seen clearly that possible differences between the hydrolysis products of RNA are quantitative rather than qualitative (equal number of fractions after separation of the hydrolysate on DEAE-cellulose, and almost identical number of spots after two-dimensional separation on filter paper). More detailed experiments undertaken with RNase *IIA* (in preparation) which represents about 60% of the nucleolytic activity in the cells of the microorganisms, show that the enzyme has a molecular weight of about 13 000 as determined by sucrose density gradient ultracentrifugation. Some of the isolated hydrolysis products have the same composition as the hydrolysis products obtained by the action of pancreatic RNase on yeast RNA [12, 11]. In view of the peculiar biological position of the *Thiobacilli*, these studies have a broad biological aspect, and will be further continued.

The authors are indebted to Miss M. Gałka for technical assistance.

SUMMARY

From cell extract of the autotrophic microorganism *Thiobacillus thioparus* three RNases, designated *IA-2*, *IIA* and *IIB* were isolated, differing with regard to optimum pH for hydrolysis of RNA, rate of thermal inactivation, and ability of adsorption on CM- and DEAE-cellulose and Amberlite IRC-50. Preliminary investigations of the mechanism of hydrolysis of RNA revealed great similarity to pancreatic RNase. The isolated RNases purified about 300-400 times in relation to the crude extract, were resistant to elevated temperature and high concentration of hydrogen ion. Acting on yeast RNA, they split off the mononucleotides, di- and trinucleotides leaving limit polynucleotides with enriched content of purine bases.

REFERENCES

- [1] Magasanik B. & Chargaff E. - *Biochim. Biophys. Acta* **7**, 396, 1951.
- [2] Markham R. & Smith J. D. - *Biochem. J.* **52**, 552, 558, 1958.
- [3] McDonald M. R., in *Biochemical Preparations* (E. E. Snell, ed.) vol. 3, p. 9. J. Wiley & Co. 1953.
- [4] Merrifield R. B. & Woolley D. W. - *J. Biol. Chem.* **197**, 521, 1952.
- [5] Ostrowski W. - *Experientia* **17**, 398, 1961.
- [6] Ostrowski W. & Walczak Z. - *Acta Biochim. Polon.* **8**, 345, 1961.
- [7] Ostrowski W. & Walczak Z., *V Internat. Congress of Biochem., Moscow 1961*. Abstr. Comm. 5. 115, vol. 9, p. 177. Pergamon Press 1963.
- [8] Plesner P. - *Acta Chem. Scand.* **9**, 197, 1955.
- [9] Rushitzky G. W. & Sober H. A. - *J. Biol. Chem.* **237**, 834, 1962.
- [10] Rushitzky G. W. & Sober H. A. - *J. Biol. Chem.* **238**, 371, 1963.
- [11] Staehelin M. - *Biochim. Biophys. Acta* **49**, 11, 1961.
- [12] Volkin E. & Cohn W. E. - *J. Biol. Chem.* **205**, 767, 1953.
- [13] Wyatt G. R. - *Biochem. J.* **48**, 584, 1951.

ENZYMY NUKLEOLITYCZNE Z *THIOBACILLUS THIOPARUS*

II. RYBONUKLEAZY I ICH WŁAŚCIWOŚCI

Streszczenie

Z ekstraktu komórek autotroficznego mikroorganizmu *Thiobacillus thioparus* wyosobniono trzy RNazy oznaczone jako IA-2, IIA i IIB, różniące się optimum pH hydrolizy RNA, szybkością inaktywacji cieplnej oraz zdolnością adsorpcji na CM-, DEAE-celulozie i na Amberlicie IRC-50. Wstępne badania nad mechanizmem hydrolizy RNA wykazują duże podobieństwo tych enzymów do RNazy trzuskowej. Wyosobnione RNazy oczyszczone w stosunku do surowego ekstraktu około 300-400 razy są odporne na działanie wyższej temperatury i na działanie dużego stężenia jonów wodorowych. Działając na RNA drożdżowy pozostawiają wielonukleotydy graniczne, znacznie wzbogacone w zasady purynowe. Poza tym w produktach hydrolizy stwierdza się mononukleotydy, dwunukleotydy i trójnukleotydy.

Received 20 December 1963.

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THE EFFECT OF METABOLIC PRODUCTS OF CYSTINE ON THE ACTIVITY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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On the basis of studies on the metabolism of sulphur amino acids Medes & Floyd [4] suggested that the disulphoxide of cystine and cystamine may be a natural metabolite in the course of oxidation of cystine. Their results, however, did not seem to provide sufficient evidence to warrant such a conclusion. At present it is generally assumed that cysteinesulphinic acid is the first stage in the oxidation of sulphur amino acids, cysteine being the substrate from which it is directly formed [2].

In our preceding study, following the suggestions of Medes & Floyd [4], we demonstrated the possibility of enzymic formation of sulfoxides by means of a model reaction between cystine and hydrogen peroxide in the presence of peroxidase [8]. We were also able to show that a compound of the type of sulfoxide formed in the course of the reaction, is an inhibitor of papain, the enzymic activity of which requires free SH groups. If in the tissues sulfoxide is formed from cystine, it could be expected that the addition of cystine to homogenates would inhibit those enzymes the activity of which depends on the presence of free SH groups.

In the present study the effect of addition of cystine to homogenates of rat muscle on the activity of glyceraldehyde-3-phosphate dehydrogenase was investigated. Pihl & Lange [6] demonstrated that in the crystalline form this enzyme is not inhibited by cystine, but its activity is diminished when cystamine monosulfoxide is added to the reaction mixture.

METHODS

Reagents. Calcium fructose-1,6-diphosphate, cysteinic acid and iodoacetamide were obtained from L. Light Co., Colnbrook, England; cystine and methylcellosolve from the Merck Co., Darmstadt, West Germany. Cystine was chromatographically pure and did not contain metal ions.

In some experiments, cystine crystallized 3 times was used and the results did not differ from those obtained with the commercial product. Cystine sulphoxide and cysteinesulphinic acid were prepared from cystine by a method which is to be published. Hydrazine, dinitrophenylhydrazine, NaOH p.a., HCl p.a., Na₂HPO₄ p.a. and trichloroacetic acid were purchased from Fabryka Odczynników Chemicznych, Gliwice, Poland.

Solutions of cystine, iodoacetamide, cystine sulphoxide, cysteinesulphinic acid, cysteinic acid and hydrazine were made in phosphate buffer of pH 7.2 (0.06 M-Na₂HPO₄ - HCl). Cystine and cystine sulphoxide were dissolved in HCl solution and then adjusted to pH 7.2 with Na₂HPO₄ solution just before adding to the proper sample. Solutions of sodium fructosediphosphate were prepared by dissolving the calcium salt in 0.03 M-HCl. Then a stoichiometric amount of oxalic acid was added, the mixture neutralized with sodium hydroxide, and calcium oxalate removed by centrifugation. To the samples to which fructosediphosphate was not added, an analogous amount of 0.03 M-HCl, previously neutralized with sodium hydroxide, was added.

Homogenates. Albino rats of Wistar strain were used. The isolated skeletal muscles were ground with quartz sand, then water was added to give a homogenate containing 1 g. of wet tissue weight per 8 ml. After centrifuging at 900 g for 10 min., the sediment was discarded and the supernatant was used for the experiments. One ml. of the homogenate was dried at 95° and dry weight was used for calculations.

Determination of glyceraldehydephosphate dehydrogenase activity. Fructosediphosphate added to the homogenate served as substrate for obtaining phosphotrioses. This procedure was possible owing to the known high activity of aldolase in the skeletal muscles of rats [7]. The activity of the dehydrogenase was determined by following the changes in phosphotriose content measured according to Lowry *et al.* [3]. The inhibitory effect of cystine and other tested compounds was assessed by the accumulation of phosphotriose. The composition of the incubation mixture is presented in the description to Fig. 1. Simultaneously samples of the same size were taken to be analysed chromatographically for the content of keto acids and aldehydes. They were added with dinitrophenylhydrazine in 2 N-HCl, the mixture was left at room temp. for 24 hr. and then extracted with toluene. The toluene solution of osazones and dinitrophenylhydrazones was concentrated in a current of air, applied marginally to a disk of Whatman no. 1 paper and chromatographed for 8 hr. in the solvent system of *n*-butanol-ethanol-0.5 N-NH₄OH (70:10:20, by vol.) [1]. After drying at room temp., the chromatograms were developed with alcoholic solution of KOH. Osazone of phosphotriose obtained by the method of Meyerhof [5] and dinitrophenylhydrazones of pyruvic acid and α -ketoglutaric acid were used as standards.

Aldolase activity of homogenates was checked by measuring the amount of phosphotriose formed and accumulated under the influence of hydrazine [7, 3].

RESULTS

Pihl & Lange [6] demonstrated that cystine has no effect on glyceraldehyde-3-phosphate dehydrogenase. Hence, the addition of cystine to the homogenates should not inhibit the activity of this enzyme. The data summarized in Fig. 1 show, however, that incubation of the homogenate with cystine (curve 5) was followed by a decrease in activity, manifested by accumulation of phosphotrioses. It seems reasonable to conclude that the observed inhibition was not due to cystine but to one of cystine transformation products. To ascertain the nature of this compound, the effect of some sulphur compounds known to be formed from cystine in homogenates of animal tissues, was examined. Those derivatives of cystine were not included which, as e.g. cysteine, are known to activate glyceraldehyde-3-phosphate dehydrogenase.

Cysteinic and cysteinesulphinic acids had no effect on the activity of the dehydrogenase (Fig. 1, curves 3, 4), as the amounts of phosphotriose were the same as in the control test (curve 2). On the other hand, cystine sulfoxide (curve 6) proved to be a strong inhibitor of the dehydrogenase, its effect being even stronger than that of iodoacetamide. The accumulation of phosphotrioses under the influence of cystine and cystine sulfoxide was dependent on the concentration of these compounds (Fig. 2).

Aldolase activity, in agreement with the findings of Siebley & Lehninger [7], was so marked that under the conditions used, the maximum phosphotriose accumulation occurred already after 3 min. of incubation. Hence, the differences shown in Fig. 1 are actually dependent only on differences in dehydrogenase activity.

Chromatographic analysis of the aldehydes and keto acids formed during incubation was in agreement with phosphotriose estimation. The chromatograms of samples with cystine, iodoacetamide and sulfoxide revealed phosphotriose osazone. Under the chromatographic conditions used, this osazone did not move from the point of application and, in contrast to most dinitrophenylhydrazones and osazones, failed to give orange colour with alkalis. However, after development with alcoholic KOH solution, it appeared as a blue spot. Besides triose, traces of the phenylhydrazone of pyruvic acid were found. The chromatograms of the control samples with cysteinic or cysteinesulphinic acid, apart from traces of the dinitrophenylhydrazone of pyruvic acid, exhibited no other spots.

For technical reasons, in our experiments instead of the direct substrate of glyceraldehyde-3-phosphate dehydrogenase, fructosediphosphate

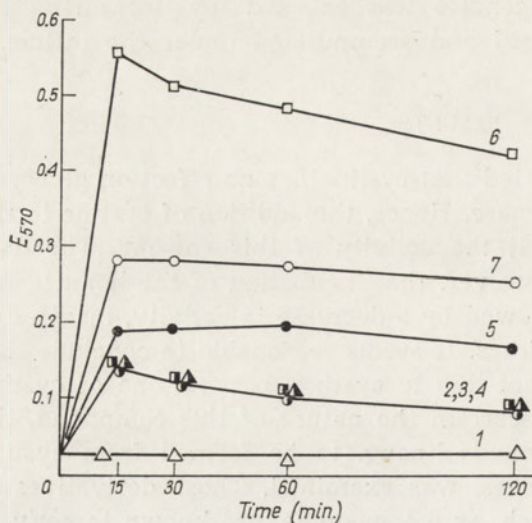


Fig. 1

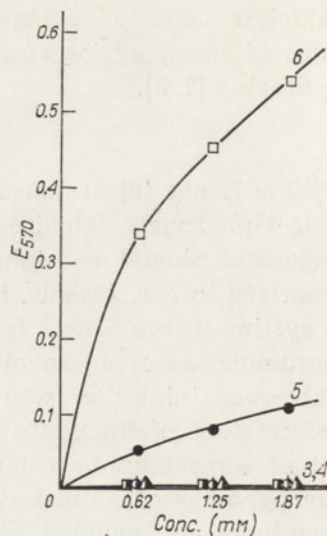


Fig. 2

Fig. 1. The effect of sulphur amino acids on glyceraldehyde-3-phosphate dehydrogenase in homogenates of rat skeletal muscles. The activity was assessed on the basis of accumulation of phosphotriose measured by the method of Lowry *et al.* [3]. To 1 ml. of the homogenate, 0.5 ml. of 16.5 mM-hexosediphosphate (final concn. 2 mM) or 0.5 ml. NaCl, and 2.5 ml. 2 mM-sulphur compound in phosphate buffer (final concn. 1.25 mM), or phosphate buffer were added. (1), NaCl, phosphate buffer; (2), hexosediphosphate, phosphate buffer; (3), cysteinesulphinic acid, hexosediphosphate; (4), cysteinic acid, hexosediphosphate; (5), cystine, hexosediphosphate; (6), cystine sulfoxide, hexosediphosphate; (7), iodoacetamide, hexosediphosphate. Samples without hexosediphosphate but containing sulphur amino acids or iodoacetamide, showed accumulation of phosphotrioses identical with that in sample no. 1, and therefore they are not represented in the diagram. The data were calculated per 25 mg. of dry weight of the homogenate.

Fig. 2. Effect of concentration of cystine (5), cystine sulfoxide (6), cysteinic acid (4), and cysteinesulphinic acid (3), on the activity of glyceraldehyde-3-phosphate dehydrogenase. Time of incubation 30 min. The data were calculated per 25 mg. of dry weight of the homogenate.

was used. Although this compound is quickly converted to phosphotriose, it may be metabolized also by other enzymes. Therefore experiments were performed to check whether the observed effect is actually the result of inhibition of the dehydrogenase and not the effect of other enzyme(s) acting on fructosediphosphate. To obtain an accumulation of phosphotrioses, in the first series of experiments hydrazine was added, and in the second series iodoacetamide, cystine or sulfoxide. Tests were also set up in which two substances causing accumulation of phosphotriose were simultaneously added (Fig. 3). In samples in which cystine was combined with sulfoxide or iodoacetamide, a summation of the

effect on accumulation of phosphotrioses was observed. On the other hand, no summation of the effect was obtained when hydrazine was combined with the other tested substances.

Since the action of hydrazine consists in binding of phosphotrioses, the absence of summation of its effect with that of sulphur compounds and iodoacetamide suggests that these substances act on another binding site than hydrazine, and affect the activity of glyceraldehyde-3-phosphate

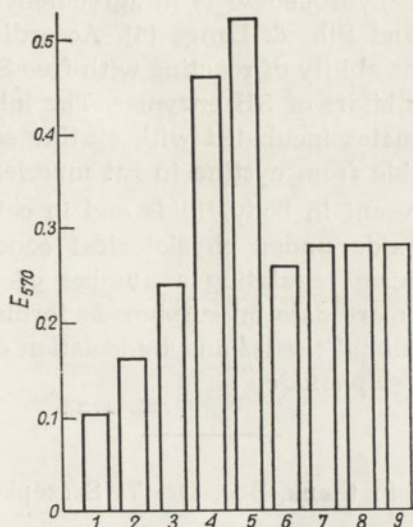


Fig. 3. The effect of cystine, cystine sulfoxide, iodoacetamide and hydrazine on the accumulation of phosphotriose in homogenates of rat muscle. To 1 ml. of the homogenate mixed with 0.5 ml. of 16.5 mM-hexosediphosphate were added the following substances dissolved in phosphate buffer: (1), 2.5 ml. of 2 mM-cystine; (2), 2.5 ml. of 2 mM-iodoacetamide; (3), 1.25 ml. each of 4 mM-iodoacetamide and cystine; (4), 2.5 ml. of 2 mM-cystine sulfoxide; (5), 1.25 ml. of 4 mM-iodoacetamide and cystine sulfoxide; (6), 2.5 ml. of 80 mM-hydrazine; (7), 1.25 ml. each of 4 mM-iodoacetamide and 160 mM hydrazine; (8), 1.25 ml. each of 4 mM-cystine and 160 mM-hydrazine; (9), 1.25 ml. each of 4 mM-cystine sulfoxide and 160 mM-hydrazine. The results are expressed as differences in the accumulation of phosphotriose between the samples and the control containing only the homogenate and hexosediphosphate.

The data were calculated per 25 mg. of dry weight of the homogenate.

dehydrogenase. These results seem to indicate that sulfoxide and cystine have no effect on other enzymes acting on fructosediphosphate. It seems also probable that sulfoxide and cystine block other SH groups of glyceraldehyde-3-phosphate dehydrogenase than does iodoacetamide.

DISCUSSION

The presented experimental findings allow to suggest that in homogenates of rat skeletal muscle cystine can be converted into a compound inhibiting the activity of glyceraldehyde-3-phosphate dehydrogenase. Of

the various compounds which can be formed from cystine in the tissues, sulphoxide had a similar effect, while cysteinic acid and cysteinesulphinic acid were without effect. Other cystine metabolites which possess free SH groups, such as cysteine, thiotaurine, alaninethiosulphonic acid and mercaptopyruvic acid, need not be taken into account as possible inhibitors of glyceraldehyde-3-phosphate dehydrogenase.

The observed effect of cystine sulphoxide on the activity of glyceraldehyde-3-phosphate dehydrogenase is in agreement with the results reported by Wills [9] and Pihl & Lange [6]. According to these authors, sulphoxides possess the ability of reacting with free SH groups of proteins and therefore are inhibitors of SH enzymes. The inhibition of the dehydrogenase in homogenates incubated with cystine seems to indicate the formation of sulphoxide from cystine in rat muscles.

Free cystine is present in body fluids and in cell cytoplasm, and its conversion to sulphoxide under physiological conditions may provide an inhibitory mechanism regulating a number of metabolic processes. It is hoped to obtain more data on sulphoxide formation in experiments performed on other animal tissues and by isolation of the enzyme which converts cystine into sulphoxide.

The authors wish to thank Doc. Dr. T. Szczepkowski for assistance and valuable advice.

SUMMARY

The effect of cystine, cysteinic acid, cysteinesulphinic acid and cystine sulphoxide on the activity of glyceraldehyde-3-phosphate dehydrogenase in homogenates of rat skeletal muscles was studied.

Cystine and cystine sulphoxide were found to inhibit the enzyme. Since it is known that cystine itself does not inhibit the crystalline dehydrogenase, its action may be explained by conversion to cystine sulphoxide in the homogenates.

REFERENCES

- [1] Bloch R. J., Durrum E. L. & Zweig G., *A manual of paper chromatography and paper electrophoresis*, p. 239, Acad. Press, New York 1958.
- [2] Greenberg D. M., *Chemical Pathways of Metabolism*. Vol. II, p. 155. Acad. Press, N. York 1954.
- [3] Lowry O. H., Roberts N. R., Wu Mei-Ling, Hixon W. S. & Crawford E. J. - *J. Biol. Chem.* **207**, 19, 1954.
- [4] Medes G. & Floyd N. - *Biochem. J.* **36**, 259, 1942.
- [5] Meyerhof O. - *Bull. Soc. Chim. Biol.* **20**, 1345, 1938.

- [6] Pihl A. & Lange R. - *J. Biol. Chem.* **237**, 1356, 1962.
[7] Siebly J. A. & Lehninger A. L. - *J. Biol. Chem.* **177**, 859, 1949.
[8] Stelmaszyńska T. & Zgliczyński J. M. - *Acta Biochim. Polon.* **10**, 371, 1963.
[9] Wills E. D. - *Biochem. J.* **63**, 514, 1956.

WPŁYW PRODUKTÓW PRZEMIANY CYSTYNY NA AKTYWNOŚĆ
DEHYDROGENAZY ALDEHYDU 3-FOSFOGLICERYNOWEGO

Streszczenie

Badano wpływ cystyny, kwasu cysteinowego, cysteinosulfinowego oraz sulfotlenku cystyny na aktywność dehydrogenazy aldehydu 3-fosfoglicerynowego zawartej w homogenatach mięśnia szkieletowego szczura.

Stwierdzono, że cystyna oraz sulfotlenek cystyny hamują badany enzym. Ponieważ wiadomo, że cystyna sama nie hamuje krystalicznej dehydrogenazy, wytłumaczono jej działanie powstawaniem z niej w homogenatach sulfotlenku cystyny.

Received 20 December 1963.

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UTILIZATION OF INFUSED PROTEIN HYDROLYSATE BY HUMAN SUBJECTS

VI. THE CONSTITUENTS OF NON-AMPHOLYTIC FRACTION OF HYDROLYSATE *

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It has been shown in many experiments that the urine excretion of bound amino acids, expressed as a percentage of infused material, is greater than that of free amino acids. However, Raczyńska-Bojanowska & Chmielewska [8] using enzymic hydrolysate of bovine blood protein containing various amounts of peptides (16 - 49% of total amino nitrogen) did not find any correlation between the amount of bound amino nitrogen introduced and excreted in the urine. These results suggested that in protein hydrolysates, bound amino acids were present in two forms, assimilable and non-assimilable, and that the differences in excretion of bound amino acids originating from various hydrolysates depended mainly on the amount of the latter form. To determine what type of bound amino acids is mainly excreted after protein hydrolysate infusion, the compounds present in the urine were separated on cationite column (H^+ form). The main fraction of extra excreted bound amino acids was present in the effluent, i.e. did not possess an ampholytic character [11, 2]. These results required closer examination of bound amino acids present in the hydrolysate used for infusion.

EXPERIMENTAL

Amino compounds of the hydrolysate were separated by column chromatography on cationite (H^+ form) and on anionite (OH^- form) columns. Following exchange resins were used: Zeo-Karb 225 (100 - 200 mesh) purified after Moore & Stein [6], and Dowex 2 X-8 (100 - 200 mesh). Analytical columns (10×1.5 cm.) and preparative columns (anionite, 20×3.0 cm., cationite, 25×4.5 cm.) were packed by sedimentation and

* Part V [11].

activated by threefold conversion from the salt into the acid or base form, and conversely. The flow rate of solutions through the columns during experiments and regeneration was 0.5 ml. and 2.0 ml. per minute for analytical and for preparative columns, respectively. Deionized distilled water was used for preparation of solutions and washing of columns.

The cationite column (H^+ form) was placed over the anionite column (OH^- form) in such a way that the effluent from the first column dropped directly into the second one. Two ml. of the hydrolysate were used for analytical, and 100 ml. for preparative separation. Compounds adsorbed on Zeo-Karb 225 were eluted with 4 M- NH_3 aq, those adsorbed on Dowex 2 with 1 N-HCl. The volumes of effluents and eluates were 50 ml. for

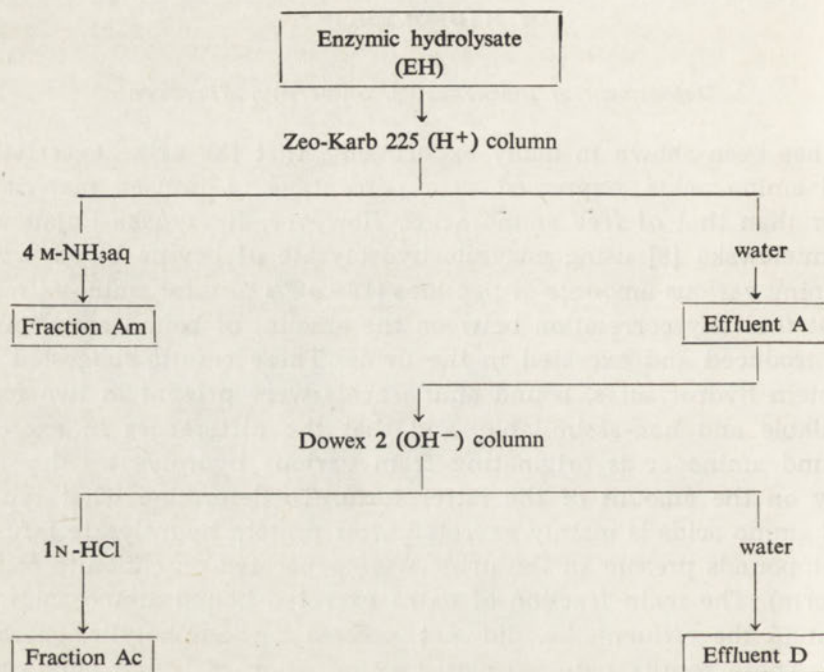


Fig. 1. Scheme of the fractionation of the constituents of blood protein enzymic hydrolysate (EH) on ion exchange columns.

analytical, and 1000 ml. for preparative fractionations. The separation diagram is given in Fig. 1. The collected fractions were evaporated in a rotatory evaporator at 20 - 25°.

α -Amino nitrogen of the free amino acids was determined by the manometric ninhydrin method of Van Slyke *et al.* [10]. Amino nitrogen was determined by the colorimetric ninhydrin method of Yemm & Cocking [13]. Acidic hydrolysis was performed with 6 N-HCl in sealed ampoules during 18 - 20 hr. at 105 - 110°. Alkaline hydrolysis was performed after Davies & Harris [3] or after Wilson & Cannan [12]. Liberated amino

acids and peptides were then separated on Zeo-Karb 225 (H^+ form) column. Synthesis of DNP-derivatives was performed according to Lockhart & Abraham [5] and determination of amino nitrogen from DNP-derivatives according to Dubin [4].

Two-dimensional paper chromatography of amino acids and peptides was performed in the systems: (I), butan-1-ol - acetic acid - water; 12:3:5, by vol. (ascending technique); (II), phenol - water, 100:25, w/w (ascending). For DNP-derivatives: (I), butan-1-ol - 1% ammonia, 1:1, v/v (ascending) and (II), 1.5 M-phosphate buffer, pH 6 (descending) were employed.

RESULTS AND DISCUSSION

The commercial preparation of bovine whole blood hydrolysate ("Aminokwas - hydrolizat pełnej krwi bydłowej", POLFA) [7] used in the experiments had an average content of total amino nitrogen 7.0 g./litre, amino nitrogen of free amino acids 5.6 g./l., and of bound amino acids 1.4 g./l. The chromatography on ion exchange resins permitted the separation of bound amino acids of the blood hydrolysate (EH) into three fractions: ampholytic (Am), non-ampholytic, acidic (Ac), and neutral (effluent D). The amounts of amino nitrogen in these fractions are given in Table 1.

Table 1

Amino nitrogen of the enzymic blood hydrolysate and of separated fractions

Determinations were made as follows: Total $N-NH_2$ in acid hydrolysate by the manometric ninhydrin method [10]; free $N-NH_2$ by the colorimetric ninhydrin method [13]; free α -amino acid $N-NH_2$ by the manometric ninhydrin method [10]. Bound $N-NH_2$ was calculated as difference between total and free α -amino acid $N-NH_2$. The results are expressed as g.N/litre enzymic hydrolysate (EH).

N-NH ₂	EH	Fraction		
		Am	Ac	Effluent D
Total	6.92	6.21	0.58	0.05
Free	5.88	5.80	0.05	—
Free α -amino acid	5.43	5.40	—	—
Bound	1.49	0.81	0.58	0.05

Further analyses were performed on fraction Ac only. After complete acid hydrolysis, paper chromatography revealed the presence of following amino acids: Glu, Gly, Asp, Pro, Ala, Ser, Thr, Leu, Val, and Cys. The first four of the listed compounds were found in greater amounts than the following ones.

On the chromatogram of the fraction Ac (developed in butan-1-

-ol - acetic acid - water) several spots were found. After spraying of the chromatogram according to Rydon & Smith [9] the most marked spots had the R_F values: 0.18, 0.31, 0.44, 0.53, 0.61, 0.66, 0.87. Some components (R_F : 0.12, 0.20, 0.28, 0.31) gave a weak reaction with ninhydrin. The component having R_F 0.20 gave also blue colour with isatin. The tests with aniline phthalate, *p*-dimethylaminobenzaldehyde and diazotized sulphanilic acid were negative for all the constituents of fraction Ac.

Some compounds present in fraction Ac gave DNP-derivatives. The quantities of amino nitrogen determined from the amount of DNP-derivatives (0.054 g./litre EH) and estimated directly by the colorimetric ninhydrin method in fraction Ac (0.050 g./litre EH) were nearly identical. Closer examinations showed that the group which reacted with fluorodinitrobenzene was not an amino group of α -amino acids. DNP-derivatives were extracted with butan-1-ol and after acid hydrolysis two DNP-compounds were found which in the system used had the R_F values different from those for all DNP- α -amino acids. In the water phase, Glu, Gly, Ala, Ser, and Leu were identified.

Table 2

Amino nitrogen of the ampholytic and non-ampholytic fractions of alkaline hydrolysates of fraction Ac

Determinations were made as described in Table 1. The results are expressed as g. N-NH₂/litre enzymic hydrolysate.

Hydrolysis temp...	37°		100°	
Fraction...	Ampholytic	Non-ampholytic	Ampholytic	Non-ampholytic
N-NH ₂ :				
Total	0.400	0.193	0.529	0.056
Free α -amino acid	0.100	—	0.350	—
Bound	0.300	0.193	0.179	0.056

Taking into account results and the isolation conditions of fraction Ac, it seems possible to characterize some general features of structure and properties of its components. It seems that apart from a few compounds reacting with ninhydrin and fluorodinitrobenzene, the fraction Ac consists mainly of peptides or amino acids with blocked amino group. One of these compounds (R_F 0.61 in butan-1-ol - acetic acid - water) was identified as pyroglutamic acid (2-pyrrolidone-5-carboxylic acid). It gave the same reactions and had identical chromatographic properties as the synthetic compound.

It was found that fraction Ac in alkaline conditions was easily hydrolysed to peptides and free amino acids which could be adsorbed on cation

exchange resin. The amounts of amino nitrogen of liberated ampholytic compounds, calculated per 1 litre of hydrolysate, are given in Table 2.

After a mild alkaline hydrolysis (17 hr. 0.3 N-NaOH, 37°) 69% of the total amino nitrogen of fraction Ac was adsorbed on Zeo-Karb 225 (H⁺ form) column. Free amino acids amounted to 25% and peptides to 75% of the adsorbed portion. In more drastic conditions (0.5 N-NaOH, 2 hr., 100°) 90% of the total amino nitrogen was adsorbed on a cationite column; free amino acids accounted for 60% of this value.

The paper chromatographic analysis of ampholytic compounds liberated from the fraction Ac in mild conditions (37°) was based on the localization of spots revealed with ninhydrin and of DNP-derivatives. On the two-dimensional chromatograms 18 ninhydrin-positive spots were found. Ten of them corresponded to amino acids identified in the acid hydrolysate, the next eight occupied the positions non-typical for α -amino acids. Chromatographic identification of DNP-derivatives soluble in ethyl ether showed the presence of DNP-derivatives of the ten amino acids identified with ninhydrin, and eight unidentified compounds. On chromatograms of the water phase (after ether extraction) two additional spots of DNP-derivatives were found.

Fraction Ac gave positive hydroxamic [14] and picric acid [1] tests. Since the synthetic diketopiperazines are also alkalilabile and give the same tests with picric acid and with hydroxylamine and ferric chloride, a cyclic structure for a part of the components of fraction Ac was suggested. However, diketopiperazines formed from monocarboxylic amino acids were not adsorbed on a Dowex 2 (OH⁻ form) column. This does not exclude a cyclic structure of the components of fraction Ac if we assume that they are formed from one molecule of dicarboxylic acid or have a side chain with a free carboxyl group.

The analysis of ampholytic compounds liberated from the components of fraction Ac by mild alkaline hydrolysis showed a considerable amount of glutamic acid. The tests with synthetic pyroglutamic acid indicated, however, that in the hydrolysis conditions applied, only 10% of pyroglutamic acid is transformed into glutamic acid; therefore the main part of this amino acid found in the alkaline hydrolysate, must originate from other compounds.

To determine the origin of the non-ampholytic, acidic components of the hydrolysate, i.e. to find out whether they are formed during enzymic hydrolysis or are due to further technological procedure, total amino nitrogen of fractions Am and Ac was estimated at different stages of production of the commercial hydrolysate (Table 3). It appeared that immediately after digestion of bovine blood protein and inactivation of the enzymes (stage A), the components of fraction Ac are present, although their quantities are insignificant as compared with those of bound amino acids of fraction Am. After decoloration of the hydrolysate

Table 3

Total amino nitrogen of enzymic hydrolysates and bound amino nitrogen of fractions Am and Ac in different stages of technological procedure

Total N-NH₂ of enzymic hydrolysate (EH) and bound of fraction Ac was determined by the manometric ninhydrin method [10]. Bound N-NH₂ of fraction Am was calculated as difference between total and free α -amino acid N-NH₂ determined by the manometric ninhydrin method [10]. Stages of production: A, after inactivation of enzymes; B, after decoloration; C, commercial product. The results are expressed as g. N-NH₂/litre of EH.

Sample	Stage	EH	Fraction		Ratio Ac:Am
			Am	Ac	
EH-1	A	9.25	2.78	0.40	1 : 7
	B	8.55	1.36	0.44	1 : 3
	C	6.32	0.79	0.54	1 : 1.5
EH-2	A	10.31	2.08	0.49	1 : 4
	B	—	—	—	—
	C	7.18	0.70	0.62	1 : 1.1
EH-3	A	9.06	1.93	0.32	1 : 6
	B	9.00	1.43	0.39	1 : 3.6
	C	6.90	0.81	0.62	1 : 1.3
EH-4	A	9.56	2.55	0.32	1 : 8
	B	—	—	—	—
	C	7.50	0.70	0.65	1 : 1.1

on carbon columns (stage B) the bound amino acids of fraction Am decreased and those of fraction Ac slightly increased. The next step, dilution with water and sterilization (stage C) resulted in further decrease of bound amino acids in fraction Am and a parallel increase in fraction Ac. It seems, therefore, that a part of bound amino acids present in fraction Ac is formed during enzymic hydrolysis, and a part results from transformation of peptides during the technological procedure.

SUMMARY

The bound amino acids present in the bovine blood hydrolysate prepared for intravenous infusion, were fractionated by ion exchange resin column chromatography. In 1 litre of the hydrolysate containing 1.4 g. of bound amino nitrogen, 0.8 g. was present in the ampholytic fraction and 0.6 g. in the non-ampholytic, acidic fraction. In the latter fraction were found: pyroglutamic acid, peptides, or amino acids bound to compounds possessing an amino group, and compounds for which the structure of cyclic diketopiperazines with a carboxyl group on a side chain seems to be most probable. Bound amino acids of the hydrolysate showing acidic and non-ampholytic properties are formed partly during enzymic hydrolysis and partly during technological processing.

REFERENCES

- [1] Abderhalden E. & Komm E. - *Z. Physiol. Chem.* **139**, 181, 1924.
- [2] Chmielewska I., Toczko K. & Szumiel I. - *Clin. Chim. Acta* **9**, 118, 1964.
- [3] Davies J. W. & Harris G. - *Biochim. Biophys. Acta* **45**, 28, 1960.
- [4] Dubin D. T. - *J. Biol. Chem.* **235**, 783, 1960.
- [5] Lockhart J. M. & Abraham E. P. - *Biochem. J.* **58**, 633, 1954.
- [6] Moore S. & Stein W. H. - *J. Biol. Chem.* **192**, 663, 1951.
- [7] Polish patent 34858, 1951.
- [8] Raczyńska-Bojanowska K. & Chmielewska I. - *Bull. Acad. Polon. Sci., Ser. Biol.* **6**, 1, 1958.
- [9] Rydon H. N. & Smith P. W. G. - *Nature* **169**, 922, 1952.
- [10] Van Slyke D. D., Mac Fadyen D. A. & Hamilton P. B. - *J. Biol. Chem.* **150**, 251, 1943.
- [11] Toczko K. & Chmielewska I. - *Acta Physiol. Polon.* **13**, 489, 1962.
- [12] Wilson H. & Cannan R. K. - *J. Biol. Chem.* **119**, 309, 1937.
- [13] Yemm E. W. & Cocking E. C. - *Analyst* **80**, 209, 1955.
- [14] Zamir A. & Lichtenstein N. - *Anal. Chim. Acta* **12**, 577, 1955.

WYKORZYSTANIE PRZEZ ORGANIZM LUDZKI SKŁADNIKÓW PODANEGO
DOŻYLNIE HYDROLIZATU BIAŁKOWEGO

VI. SKŁADNIKI NIEAMFOLITOWEJ FRAKCJI HYDROLIZATU

Streszczenie

Składniki azotowe hydrolizatu białkowego do wlewań dożylnych Aminokwasy - hydrolizat krwi bydłowej, POLFA, frakcjonowano na żywicach jonowymiennych. Zawartość związanego azotu aminowego w litrze preparatu wynosi 1.4 g., z czego związki o charakterze amfolitowym stanowią 0.8 g. a związki kwaśne, nieamfolitowe 0.6 g. We frakcji nieamfolitowej znaleziono kwas piroglutaminowy, peptydy (lub aminokwasy) dołączone do związków zawierających grupę aminową, lecz nie będących α -aminokwasami oraz szereg związków, dla których najbardziej prawdopodobna wydaje się budowa cykliczna dwuketopiperazynowa z grupą karboksylową w łańcuchu bocznym. Część aminokwasów związanych hydrolizatu, posiadających własności kwasowe i nieamfolitowe, tworzy się w czasie hydrolizy enzymatycznej, część w czasie obróbki technologicznej preparatu.

Received 21 December 1963

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**L-3-ALDONIC ACID DEHYDROGENASE FROM
*SCHWANNIOMYCES OCCIDENTALIS***

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Dedicated to the memory of the late Professor Bolesław Skarżyński

In recent papers dealing with the catabolism of *meso*-inositol in the yeast *Schwanniomyces occidentalis* [5, 6], it has been reported that this organism contains an enzyme or enzyme system catalysing the oxidative decarboxylation of L-gulonic acid to L-xylulose. The same catabolic reaction exists also in animal tissues. In a detailed study of the enzymes concerned, Smiley & Ashwell [7] have shown that the conversion of L-gulonic acid to L-xylulose in hog kidney proceeds *via* 3-keto-L-gulonic acid as intermediate. A NAD-specific dehydrogenase dehydrogenates L-gulonate to 3-keto-L-gulonate; this enzyme shows a somewhat broader specificity catalysing also the dehydrogenation of several other 3-L-hydroxyacids. Therefore, the authors propose to name the enzyme β -L-hydroxyacid dehydrogenase; the corresponding systematic name should be L-3-hydroxyacid:NAD oxidoreductase [3]. Another enzyme, a 3-keto-L-gulonate decarboxylase (systematic name: 3-keto-L-gulonate carboxyl-lyase) transforms the intermediate to L-xylulose [9].

It seemed of interest to study the enzyme system responsible for the oxidative decarboxylation of L-gulonic acid to L-xylulose in *Schwanniomyces occidentalis* in order to see whether the enzymes catalysing this transformation in this yeast are analogous to those present in hog kidney.

The present paper deals with the partial purification and with the characterization of the enzyme dehydrogenating L-gulonic acid in *Schwanniomyces occidentalis*. Although this enzyme is also NAD-specific and the product of its action on L-gulonic acid is 3-keto-L-gulonic acid, it differs in some respects from that found in animal tissues.

MATERIALS AND METHODS

Materials. L-Gulonolactone, sodium D-gluconate, D-xylose and clupeine sulphate were purchased from Fluka, Buchs (Switzerland). The tetrazoliumchloride blue was obtained from Loba-Chemie, Vienna. L-Xylonolactone, L-galactonolactone, calcium L-idonate, barium D-lyxonate, and L-xylulose were generous gifts of Dr. G. Ashwell, Bethesda, Maryland. To obtain the free acids, the lactones were hydrolysed with 1 N-NaOH at room temperature or with 0.1 N-NaOH at 60°; for the paper chromatography the kations were removed with kation exchange resins and the resulting solutions evaporated to dryness. Sodium acetoacetate was obtained from the ester by hydrolysis [4].

Culture of the organism. The strain of *Schwanniomyces occidentalis* used for the enzyme preparations was obtained from the collection of the Institut für biochemische Technologie und Mikrobiologie of the Technische Hochschule, Vienna. It was cultured on the basal medium of Reader [2] to 1 liter of which 10 g. of meso-inositol and the mixture of growth factors in the proportions of Wickerham & Burton [8] were added. The Reader-medium, the solution of meso-inositol, and the mixture of growth factors

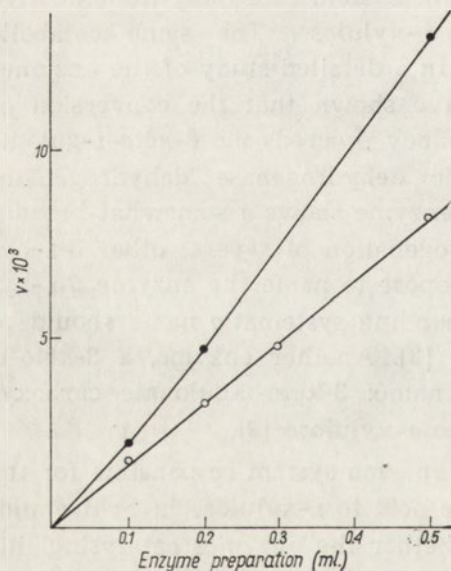


Fig. 1. Dependence of the reaction rate on the concentration of enzyme. The test solution contained 100 μ moles L-gulonic acid (○) or D-gluconic acid (●), 1.5 μ moles NAD, 100 μ moles tris buffer, pH 9, and varying amounts of enzyme in a total volume of 3 ml. Measurements were made 10 min. after the addition of the enzyme in a Beckman model DU spectrophotometer.

were sterilized separately. The cultures were grown on an agar medium containing 15 g. agar per liter. In order to obtain larger amounts of cells for the preparation of the enzyme, the agar culture was transferred to 80 ml. of the medium described and agitated for 3 days. Then this culture was transferred to 5 liters of the same medium and was well aerated for

3 days at 30°. Finally, the cells were harvested by centrifugation; the yield was 35 to 40 g. wet weight, corresponding to about 4 g. dry weight.

Assay procedure. The activity of the enzyme was routinely measured with L-gulonate and NAD as substrates. The rate of NAD reduction was determined by measuring the increase of optical density at 340 m μ . Detailed conditions for the assay are described in the legend to Fig. 1.

Paper chromatography of the products. For the separation and identification of the acid and neutral compounds formed from L-gulonate, four solvent systems were used: (1), the upper layer of the mixture of butanol-ethanol-water (5:1:4, by vol.), descending technique; (2), butanol-acetic acid-water (4:1:1, by vol.), descending technique; (3), the upper layer of the mixture of ethylacetate-pyridine-water (2:1:2, by vol.), ascending technique; due to aging processes no constant R_F values in this system could be obtained; (4), acetone-water (9:1, v/v), ascending technique. The R_F values of the various compounds are given in Table 1.

Table 1

R_F values of various sugars and sugar acids in the solvent systems used

Solvent systems as described under Materials and Methods. Spray reagent: ammoniacal silver nitrate solution. In the system no. 3 the R_F values change with the age of the solvent system; it is always necessary to work with control substances. For the system no. 4 the $R_{xylulose}$ values are given.

Substance	Solvent system				Tetrazolium blue test
	1	2	3	4	
Xylulose	0.27	0.28	0.38	1.00	++
Xylose	0.21	0.21	0.245	0.73	+
Lyxose	0.23	—	0.24	—	(+)
Ascorbic acid	—	0.37	—	—	++++
Glucuronic acid	0.03	0.14	0.01	—	(+)
Glucuronolactone	0.30	0.29	0.68	—	+
L-Gulonic acid	0.06	0.13	0.05	—	—
L-Gulonolactone	0.19	0.19	0.47	—	—
L-Galactonolactone	0.265	0.25	0.60	—	—
L-Idonic acid	0.04	0.125	—	—	—
L-Idonolactone	0.28	0.28	—	—	—
D-Gluconic acid	0.04	0.13	—	—	—
D- γ -Gluconolactone	0.32	0.33	—	—	—
D- δ -Gluconolactone	0.16	0.21	—	—	—

Enzyme purification

Crude extract. 10 g. (wet weight) of the harvested cells of *Schwanomyces occidentalis* in 0.025 M-tris buffer solution, pH 9.1, were disrupted in the cell disintegrator of Merckenschlager *et al.* [1]. The homogenate was centrifuged 30 min. at 15 000 *g* and the residue discarded.

Clupeine sulphate. The supernatant (crude extract) was brought to pH 7.4 by addition of 0.5 N-H₂SO₄. Then a solution of 0.1 g. clupeine sulphate was added. After 15 min. the precipitate was removed by centrifugation and discarded.

Ammonium sulphate. To the solution 0.1 M-KCN was added until a pH of 8.0 was reached, then finely powdered ammonium sulphate was added to 50% saturation. After 30 min. standing the precipitate was removed by centrifugation at 15 000 g. The supernatant solution was added with ammonium sulphate to 60% saturation and was left standing in the cold for 12 hr. The supernatant which contained much protein but almost no enzyme activity was rejected after centrifugation at 15 000 g. The precipitate was dissolved in a mixture of 3 ml. 0.025 M-tris buffer, pH 9.0, 1 ml. H₂O, and 0.5 ml. 0.1 M-KCN.

Sephadex G 100. The solution was placed on a column of Sephadex G 100 (1.8 × 33 cm.). The enzyme was eluted with 0.005 M-tris buffer, pH 9.0, which was 0.0005 M with respect to KCN; it appeared as a distinct fraction after 40 ml. of the eluting agent have passed the column. No loss of enzyme activity was observed in this step even when working at room temperature.

Calcium phosphate gel. To the eluate one tenth of its volume of calcium phosphate gel (containing 11 mg. calcium phosphate per ml.) was added. After standing for 15 min. the residue which contained some protein but no enzyme activity was rejected. Then the supernatant was brought to pH 6.3 with aqueous phosphoric acid and once again one tenth of its volume of calcium phosphate gel was added. After centri-

Table 2

Summary of purification procedure

The activity is expressed in international units (U) [3].

Fraction	Total activity (U)	Specific activity m-units/mg. protein	Yield (%)
Homogenate (crude extract)	2.48	2.85	(100)
Clupeine sulphate 0.01 %	2.34	15.5	94
Ammonium sulphate 50 - 60 % saturation	1.95	23.6	79
Sephadex G 100	1.85	52	74
Calcium phosphate gel, pH 9.0	1.85	67.5	74
Calcium phosphate gel, pH 6.3	1.67	108	67

fugation the supernatant was discarded. The gel was extracted with 5 ml. 0.02 M-tris buffer which was 0.02 M with respect to KCN.

All steps of this purification procedure were carried out in the cold. A summary of the purification steps is given in Table 2.

Identification of the product of the dehydrogenation of L-gulonate by the enzyme

A solution of 50 mg. NAD, and 1.1 g. sodium L-gulonate were dissolved in 18 ml. of water. To this solution 10 ml. tris buffer, pH 9.1, and 2 ml. enzyme solution (specific activity 108 milliunits per ml.) were added and the mixture was incubated for 2 hr. at 30°. The solution thus obtained was concentrated to a volume of 4 ml. by distillation *in vacuo*, then centrifuged and the residue discarded. The supernatant was brought to pH 7.5 and then first placed on a column (3 × 17 cm.) containing Dowex 1 × 8 (formate form), and afterwards on a column (2 × 13 cm.) of Dowex 50 W (200 - 400 mesh, H⁺ form). The resulting eluate was evaporated and the residue analysed by paper chromatography. This neutral fraction contained L-xylulose, xylose, and some L-gulonic acid which had passed the column as lactone.

The Dowex 1 column was first washed with water and then eluted with 1 N-HCOOH. The eluate was collected in 30 ml. fractions each of which was examined by paper chromatography. Those fractions which contained keto acids, i.e. spots giving a positive tetrazolium test, were further investigated.

To characterize the keto acid obtained, the solution in which it was present was brought to pH 7.0 by addition of NaOH, and then placed into the vessel of a pH-stat (Radiometer). While the pH was kept constant with HCl, a solution of 20 mg. NaBH₄ in 1 ml. of water was slowly added through a burette [cf. 10]. The mixture containing the products of the borohydride reduction was acidified with HCl, evaporated to dryness, the dry residue dissolved in water and once again evaporated to dryness. This time the residue was extracted with hot absolute ethanol. After evaporation of the ethanol, the residue was again dissolved in water and placed first on a column of Dowex 50 W, as above, and then on a column (1.5 × 9 cm.) of Dowex 2 × 8 (200 - 400 mesh, Cl⁻ form). The solution thus obtained was evaporated and the residue analysed by paper chromatography. On the paper two spots could be detected which by comparison with authentic samples could be identified with L-gulonic acid and L-galactonic acid. This seems to prove that the product of the enzyme action on L-gulonic acid is 3-keto-L-gulonic acid, because only this compound can be reduced to the epimeric mixture mentioned above.

Properties of the enzyme

Stability. In solutions the enzyme is rather instable. The stability can, however, be greatly improved by the addition of cyanide which acts in concentrations below 10^{-3} M in all steps of the purification procedure. Under the protecting influence of cyanide the enzyme preparations can be kept in the cold for several days without decrease of the activity. A minor stabilizing effect is also observed when EDTA is added to the enzyme preparations; neither cysteine nor NAD show any stabilizing action.

Specificity of the enzyme. The purified enzyme shows activity towards aldonic acids which contain a 3-hydroxyl group in L-configuration. In this respect, the specificity of our enzyme is analogous to that of the hog kidney enzyme of Smiley & Ashwell [7]. However, purified preparations of our enzyme did not dehydrogenate L- β -hydroxybutyric acid which is attacked by the animal enzyme. Also the reverse reaction, the hydrogenation of acetoacetate to β -hydroxybutyric acid with NADH as hydrogen donor could not be demonstrated. It should be mentioned that less purified preparations of our enzyme are capable of dehydrogenating β -hydroxybutyric acid which indicates that this activity is due to an enzyme impurity rather than to the enzyme active toward L-gulonic acid and the other L-3-hydroxy sugar acids. As can be seen in Table 3, aldonic acids with a D-3-hydroxy configuration are not attacked at all by our enzyme.

Table 3

Relative rate of oxidation of some 3-hydroxyacids by the enzyme from Schwanniomyces occidentalis

Substrate	Relative activity, as compared to the rate of oxidation of L-gulonic acid	
L-Gulonate	100	(100)
D-Gluconate	125	(49)
D-Lyxonate	130	(81)
L-Idonate	160	(59)
Hydroxybutyrate	0	(26)
L-Galactonate	0	(0)
D-Altronate	0	(0)
L-Xylonate	0	(0)
Serine	0	
Threonine	0	

pH-Optimum. The dehydrogenation of L-gulonic acid by the enzyme shows a rather sharp pH-optimum at 9.0 in 0.1 M-glycine-NaOH buffer.

Michaelis constants. The Michaelis constants of the enzyme towards

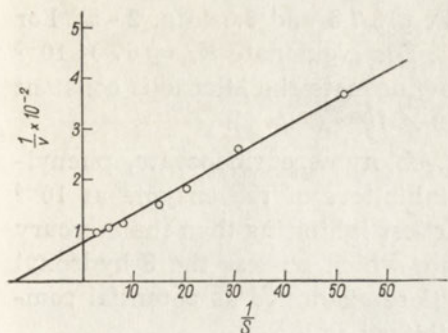


Fig. 2

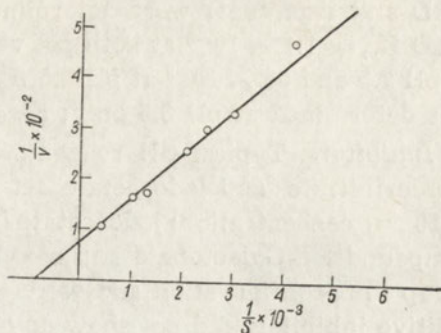


Fig. 3

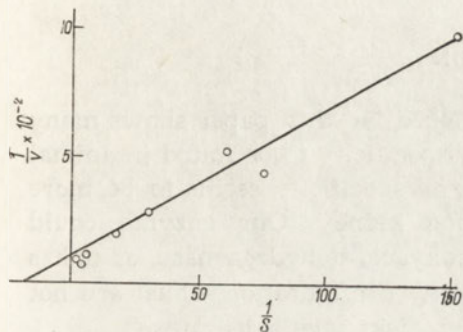


Fig. 4

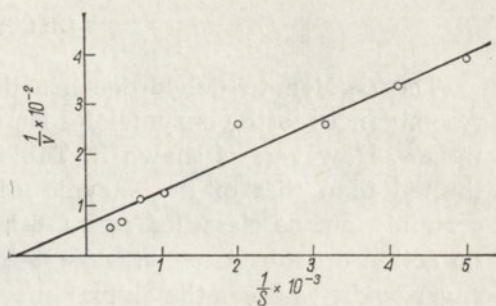


Fig. 5

Fig. 2. Lineweaver-Burk diagram for the determination of the Michaelis constant for L-gulonate at pH 7.5. The reaction mixture contained 100 μ moles tris buffer, pH 7.5, 1.5 μ moles NAD, 0.1 ml. enzyme solution (1.2 mg. protein/ml.) and varying amounts of L-gulonate; total volume 3 ml.

Fig. 3. Lineweaver-Burk diagram for the determination of the Michaelis constant for NAD at pH 7.5. The reaction mixture contained 100 μ moles tris buffer, pH 7.5, 100 μ moles of sodium-L-gulonate, 0.1 ml. enzyme solution (1.2 mg. protein/ml.) and varying amounts of NAD; total volume 3 ml.

Fig. 4. Lineweaver-Burk diagram for the determination of the Michaelis constant for L-gulonate at pH 8.5. Conditions, except the buffer solution, as in Fig. 2.

Fig. 5. Lineweaver-Burk diagram for the determination of the Michaelis constant for NAD at pH 8.5. Conditions, except the buffer solution, as in Fig. 3.

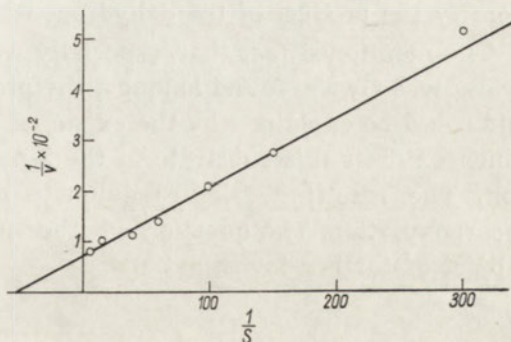


Fig. 6. Lineweaver-Burk diagram for the determination of the Michaelis constant for D-gluconate at pH 8.5. Conditions as in Fig. 2, except that varying amounts of D-gluconate were used instead of L-gulonate.

NAD and L-gulonate were determined at pH 7.5 and 8.5 (Fig. 2-5). For NAD $K_m = 1.1 \times 10^{-3}$ at both pH values. For L-gulonate $K_m = 67 \times 10^{-3}$ at pH 7.5 and 55×10^{-3} at pH 8.5. For D-gluconate the Michaelis constant was determined at pH 8.5 only; $K_m = 19 \times 10^{-3}$ (Fig. 6).

Inhibitors. Typical SH reagents as p-chloromercuribenzoate, phenylmercurinitrate, and iodoacetate act as inhibitors of the enzyme at 10^{-4} to 10^{-3} M concentration; iodoacetate is far less inhibiting than the mercury compounds. L-Galactonate and D-xylonate which possess the 3-hydroxyl group in D-configuration and were therefore examined as potential competitive inhibitors did not show any inhibitory action.

DISCUSSION

The L-gulonate dehydrogenase described in this paper shows many resemblances to the enzyme of a similar specificity to be found in animal tissues. However, as shown in Table 3, its specificity seems to be more limited than that of the enzyme of hog kidney. Our enzyme could certainly not be classified as a L-3-hydroxyacid dehydrogenase, as it has no action on substrates with a L-3-hydroxy configuration which are not sugar acids, whereas the animal enzyme attacks such substrates.

As for the biological role of the enzyme, it could be deduced from our previous work [5, 6] that its main function lies in the degradation of L-gulonic acid which is an intermediate in the catabolism of meso-inositol and D-glucuronic acid in the studied organism. We also have good evidence that an enzyme exists in *Schwanniomyces occidentalis* which catalyses the decarboxylation of 3-keto-L-gulonic acid, the product of the action of the dehydrogenase on L-gulonic acid, to L-xylulose. This decarboxylase will be described in a further communication.

Some doubts are, however, cast on the assumption that the action on L-gulonic acid is the main function of the described dehydrogenase by the fact that its affinity towards L-gulonic acid is relatively low in comparison with that towards D-gluconic acid. So far, we have not yet been able to identify the product of the dehydrogenation of D-gluconic acid.

The mentioned fact that, especially with impure enzyme preparations, xylose was always found amongst the products of the action on L-gulonic acid could be explained by the existence of an enzyme in the preparation which oxidizes this substrate in the 2-position rather than in the 3-position. The resulting 2-keto-L-gulonate could give rise to L-xylose by decarboxylation. The question whether such an enzyme exists will be the subject of further investigations.

We are grateful to Dr. G. Ashwell, Bethesda, Maryland, for his valuable discussion and for a generous gift of several rare aldonic acids. This study was supported by a grant from the Ludwig-Boltzmann-Gesellschaft, Vienna.

SUMMARY

A NAD-specific dehydrogenase oxidizing L-gulonic acid to 3-keto-L-gulonate has been found in the yeast *Schwanniomyces occidentalis*. After a partial purification it could be shown that the enzyme is active on aldonic acids having the L-3-hydroxy configuration. Although its specificity is somewhat more limited, the enzyme resembles in many respects to the β -L-hydroxyacid dehydrogenase of hog kidney.

REFERENCES

- [1] Merckenschlager M., Schlossmann K. & Kurz W. - *Biochem. Z.* **329**, 332, 1957.
- [2] Reader V. - *Biochem. J.* **21**, 904, 1927.
- [3] *Report of the Commission on Enzymes of the International Union of Biochemistry*. Pergamon Press, Oxford 1961.
- [4] Seeley H. W., in *Methods of Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. I, p. 624. Pergamon Press, New York 1955.
- [5] Sivak A. & Hoffmann-Ostenhof O. - *Biochim. Biophys. Acta* **53**, 426, 1961.
- [6] Sivak A. & Hoffmann-Ostenhof O. - *Biochem. Z.* **336**, 229, 1962.
- [7] Smiley J. D. & Ashwell G. - *J. Biol. Chem.* **236**, 357, 1961.
- [8] Wickerham L. J. & Burton A. K. - *J. Bact.* **56**, 363, 1948.
- [9] Winkelman J. & Ashwell G. - *Biochim. Biophys. Acta* **52**, 170, 1961.
- [10] Wolfrom M. L. & Anno K. - *J. Am. Chem. Soc.* **74**, 5583, 1952.

DEHYDROGENAZA KWAŚÓW L-3-ALDONOWYCH ZE *SCHWANNIOMYCES OCCIDENTALIS*

Streszczenie

W drożdżach *Schwanniomyces occidentalis* stwierdzono obecność współpracującej z NAD dehydrogenazy, która utlenia kwas gulonowy do 3-keto-L-gulonowego. Po częściowym oczyszczeniu stwierdzono, że enzym utlenia i inne kwasy aldonowe posiadające na C₃ konfigurację L. Enzym pod wielu względami wykazuje podobieństwo do dehydrogenazy β -L-hydroksykwasów z nerki wieprza, jednak jego specyficzność jest nieco bardziej ograniczona.

Received 28 December 1963.

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THE FIVE-MEMBERED DISULPHIDE RING SYSTEM

III. ANTINEOPLASTIC POTENTIALITIES **

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Various authors have shown that α -lipoic acid and similar cyclic disulphides may have a profound effect on growth and metabolism of a variety of cells and organisms. In two previous publications [8, 15] we have reviewed the literature and reported on the physico-chemical properties [15] and the biological activity [8] of a large number of α -lipoic acid analogues, which were shown to be active inhibitors of the growth of clone 929 of Earle's strain L mouse fibroblasts grown *in vitro*. All of these analogues are 1:2-dithiolane derivatives containing the five-membered disulphide ring of lipoic acid. However, in most of the compounds studied a side chain was attached to the disulphide ring at the fourth and not at the third position (see Table 1).

When the cytostatic effects of these 1:2-dithiolane derivatives were compared at equimolar concentrations the most potent inhibitors were found to be those with a semicarbazone side chain. 2:3-Dithio-4:5-spiro-decane was also very active, while the attachment of two hydroxymethyl groups at the fourth position yielded an agent with only a moderate cytostatic effect. The 4-carbonic acid derivative caused a considerable growth inhibition, while the effect of 1:2-dithiolane-3:5-dicarboxic acid was less pronounced. However, if the cytostatic effects of these analogues were compared at concentrations corresponding to the LD₅₀ dose in mice the 3:5-dicarboxic acid derivative was found to be among the most potent inhibitors, and also 4:4-bishydroxymethyl-1:2-dithiolane showed considerable activity. A number of other agents with a low solubility were found to have little or no cytostatic effect.

* Visiting Research Fellow on leave of absence from the Polish Academy of Sciences, Department of Experimental Pathology, Warsaw, with the support of the Danish Cancer Society, The Irma Foundation and Tømrermester Jørgen Holm's Foundation.

** Parts I and II were published in Biochemical Pharmacology (see ref. [15] and [8]).

Table 1
Some 1:2-dithiolane derivatives

Compound	Code no.	Formula
α -Lipoic acid	—	$ \begin{array}{c} \text{S} - \text{S} \\ \quad \\ \text{CH}_2 \quad \text{CH} - (\text{CH}_2)_4 - \text{COOH} \\ \diagdown \quad \diagup \\ \text{CH}_2 \end{array} $
1:2-Dithiolanon-4-semicarbazone	Ph 800/1	$ \begin{array}{c} \text{S} - \text{S} \\ \quad \\ \text{CH}_2 \quad \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{C} = \text{N} - \text{NH} - \text{C}(=\text{O}) - \text{NH}_2 \end{array} $
1:2-Dithiolanon-4-thiosemicarbazone	Ph 800/18	$ \begin{array}{c} \text{S} - \text{S} \\ \quad \\ \text{CH}_2 \quad \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{C} = \text{N} - \text{NH} - \text{C}(=\text{S}) - \text{NH}_2 \end{array} $
2:3-Dithio-4:5-spirodecane	Ph 800/2	$ \begin{array}{c} \text{S} - \text{S} \\ \quad \\ \text{CH}_2 \quad \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{CH}_2 \quad \text{CH}_2 \\ \quad \\ \text{CH}_2 \quad \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{CH}_2 \end{array} $
4:4-Bishydroxymethyl-1:2-dithiolane	Ph 800/7	$ \begin{array}{c} \text{S} - \text{S} \\ \quad \\ \text{CH}_2 \quad \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{C} \\ \diagup \quad \diagdown \\ \text{CH}_2\text{OH} \quad \text{CH}_2\text{OH} \end{array} $
1:2-Dithiolane-4-carbonic acid	Ph 800/21	$ \begin{array}{c} \text{S} - \text{S} \\ \quad \\ \text{CH}_2 \quad \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{CH} - \text{COOH} \end{array} $
1:2-Dithiolane-3:5-dicarbonic acid	Ph 800/8	$ \begin{array}{c} \text{S} - \text{S} \\ \quad \\ \text{HOOC} - \text{CH} \quad \text{CH} - \text{COOH} \\ \diagdown \quad \diagup \\ \text{CH}_2 \end{array} $

The demonstration of the growth inhibitory effect of α -lipoic acid led various authors to a study of the antineoplastic potentialities of this agent [3, 4, 10], but no tumour inhibiting activity could be demonstrated. Similarly, with one exception, we were unable to demonstrate any inhibitory effect of lipoic acid analogues on the growth of a hypotetraploid subline of Ehrlich's ascites tumour *in vivo*. However, our experiments indicated that these negative results were due to rapid elimination of the drugs from the abdominal cavity rather than to cellular resistance.

In order to gain further information about the antineoplastic potentialities of the 1:2-dithiolane derivatives their effect on ascites tumour cells was studied in greater detail *in vitro* as well as *in vivo*. Parallel investigations of their cytostatic effect on L-929 mouse fibroblasts were carried out simultaneously for comparison.

MATERIAL AND METHODS

The cells studied were Landschütz hyperdiploid line of Ehrlich's ascites carcinoma (ELD), which was propagated *in vivo* in mice of the DBA/212 strain by intraperitoneal transplantations and *in vitro* in flasks with Eagle's minimum essential medium [5] with double concentration of glutamine and with $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (0.42 mM) and KH_2PO_4 (0.44 mM) instead of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.81 mM) instead of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. This medium was fortified with 10% calf serum. The ELD-cells were originally adapted to *in vitro* conditions by dr. Holmberg of the Research Division at Radiumhemmet, Stockholm. They have been grown in culture at the Fibiger Laboratory for almost two years.

The growth studies also included Earle's strain L-929 mouse fibroblasts, which have been propagated at this laboratory for 7 years. In the present experiments these cells were grown under the same conditions as the ELD-cells.

For the study of the cytostatic effect *in vitro* of various 1:2-dithiolane derivatives, cultures were prepared from cell suspensions containing 100 000 - 250 000 cells per ml. After incubation for 24 hr. in the control medium an initial cell count was made of ten cultures selected at random. The cells to be counted were trypsinized for 10 min. with a 1.25% trypsin solution and counted in a Bürker-Türk haemocytometer.

The remaining cultures were divided into groups of ten and treated with varying concentrations of the compound under investigation. The medium was changed daily, and after 3 - 6 days a final count was made of all cultures. The median values and the 95% confidence interval were calculated as described by Dean & Dixon [1].

Numerous experiments were carried out in order to elucidate the effect of a large number of doses of the various compounds. The highest

dose causing a significant, but incomplete growth inhibition and the lowest dose showing complete growth inhibition in all experiments indicated the interval within which the minimal dose required for total growth inhibition (min. ID₁₀₀) would be found.

The cytostatic effect *in vivo* of three selected α -lipoic acid analogues on the ELD-cells was also studied. Ascites tumour cells obtained from a mouse seven days after transplantation were suspended in Tyrode's solution and reinoculated into mice of the DBA/212 strain at a dose of $1-5 \times 10^6$ cells per animal. After nine days the animals were killed. The ascitic fluid was carefully withdrawn and the abdominal cavity was washed twice with a total of 5 ml. of Tyrode's solution. All the cells thus removed from each individual animal were pooled and counted.

The cytocidal effect of the selected 1:2-dithiolane derivatives was investigated by exposing ELD-cells obtained from animals and cultures to the compounds *in vitro* for 8-72 hr. The derivatives were dissolved in Eagle's medium at a concentration corresponding to $1/5$ LD₅₀. After this treatment the viability of cells obtained from animals was tested by reinoculation of 2.5×10^6 cells intraperitoneally (i.p.) into each of ten mice of the DBA/212 strain. The viability of cells obtained from cultures was tested by studying their ability to grow after replacement of the cytostatic medium with normal medium. The total incubation period was 6 days, so that the cells had at least 3 days for recovery before they were trypsinized and counted.

The antimitotic effect was studied in cultures of ELD-cells grown on flying coverslips. After exposure to the various compounds for 8 hr. the cells were fixed in ether-alcohol and stained with May-Grünwald-Giemsa stains. The number of mitotic figures per 1000 cells was counted in each culture.

Respiratory studies were carried out with conventional Warburg technique. The cells were suspended in Ringer-Locke's solution buffered with tris(hydroxymethyl)aminomethane at a concentration of 30 mM. In many experiments glucose was omitted from the solution and in others replaced by lactate, α -ketoglutarate or oxaloacetate at a concentration of 5 mM. All substrates were purchased from Sigma Chemical Company, St. Louis, Missouri, USA.

All the α -lipoic acid analogues were generously supplied by Pharmacia, Uppsala, while α -lipoic acid was obtained from Chemiewerk, Homburg, Frankfurt/Main.

RESULTS

Cytostatic effect in vitro. The cytostatic effect of 1:2-dithiolane derivatives on cells grown *in vitro* was studied as described. The results are summarized in Table 2, which shows the dosage intervals within

which the minimal doses required for total inhibition of the growth of the L-929 fibroblasts and the ELD-carcinoma cells were found. As it is seen the ELD-cells were more sensitive to several of these compounds than the L-929 fibroblasts. The Table also shows the LD₅₀ values in mice determined by single intraperitoneal injection, at Pharmacia, Uppsala. The figures suggest that several 1:2-dithiolane derivatives may inhibit the growth of Ehrlich's ascites tumour at doses tolerated by the host.

In Table 3 are shown the results of an experiment, in which the cytostatic effect of six α -lipoic acid analogues on the L-929 fibroblasts and the ELD-cells was studied *in vitro* at concentrations corresponding to 20% of the LD₅₀ dose. The two semicarbazone derivatives (Ph 800/1 and Ph 800/18) as well as the dicarbonic acid derivative (Ph 800/8)

Table 2

Cyto-toxicity and general toxicity of 1:2-dithiolane derivatives

LD₅₀ determinations were made by single i.p. injection at Pharmacia, Uppsala.

Compound	ELD-cells		L-Fibroblasts		Mice
	Min. ID ₁₀₀ (m-moles/liter)				LD ₅₀ (m-moles/kg.)
	>	<	>	<	
Lipoic acid	0.50	0.75	1.50	3.00	3.00
Ph 800/1	0.01	0.02	0.02	0.04	0.19
Ph 800/18	0.02	0.03	0.035	0.07	0.34
Ph 800/2	0.04	0.08	0.04	0.08	0.78
Ph 800/7	0.35	0.70	1.50	3.00	0.72
Ph 800/21	0.10	0.20	0.60	0.80	0.18
Ph 800/8	0.75	1.50	1.50	3.00	15.00

Table 3

The cytostatic effect of 1:2-dithiolane derivatives on the ELD-tumour cells and the L-929 fibroblasts in vitro at concentrations corresponding to 1/5 LD₅₀

Median values with 95% confidence interval [1] are given.

Compound	Concn. (mM)	Cell number (millions per culture)			
		ELD-cells		L-929 fibroblasts	
		Initial	After 4 days	Initial	After 4 days
Control	—	0.515 ± 0.069	3.780 ± 1.432	1.190 ± 0.285	3.640 ± 0.460
Ph 800/1	0.038	—	0.100 ± 0.060	—	0.040 ± 0.124
Ph 800/18	0.068	—	0.070 ± 0.037	—	0.640 ± 0.302
Ph 800/2	0.156	—	0.505 ± 0.288	—	1.240 ± 0.670
Ph 800/7	0.144	—	4.400 ± 0.700	—	2.760 ± 0.832
Ph 800/21	0.036	—	3.010 ± 0.782	—	2.580 ± 0.607
Ph 800/8	3.000	—	0.072 ± 0.012	—	1.580 ± 0.364

inhibited cell growth completely. Also 2:3-dithio-4:5-spirodecane (Ph 800/2), which is a water insoluble liquid, had a pronounced effect, while the remaining two compounds were inactive at these concentrations. This is in agreement with the results shown in Table 2. The three first mentioned analogues, which were the most active of the water soluble compounds, were selected for further studies.

Cytocidal effect in vitro. The demonstration of a cytostatic effect of the 1:2-dithiolane derivatives does not necessarily indicate that these compounds kill the cells. Therefore, the cytocidal effect of the three selected analogues was studied as described. It appears from Table 4 that the transplantation test failed to disclose any cytocidal effect after 8 hr. of preincubation. After 24 hr. the semicarbazone derivatives showed a cytocidal effect, while it took 72 hr. of preincubation before the dicarbonic acid derivative caused any reduction of the percentage of takes.

Table 5 shows the ability of the ELD-cells to grow after treatment for varying lengths of time. As it is seen the cytostatic effect of

Table 4

The effect of 1:2-dithiolane derivatives on the transplantability of ELD-tumour cells

Compound	Duration of preincubation (hr.)			
	8	24	48	72
	Percentage of takes			
Controls	100	100	100	100
Ph 800/8 (3 mM)	100	100	100	70
Ph 800/1 (0.038 mM)	100	10	0	0
Ph 800/18 (0.068 mM)	100	0	0	0

Table 5

The cytocidal effect of Ph 800/8, Ph 800/1 and Ph 800/18 on the ELD-tumour cells in vitro

Median values with 95% confidence interval [1] are given.

Compound concn.	Initial no. of cells (millions per culture)	Cell number (millions per culture after 6 days)				
		Duration of treatment (hr.)				
		Controls	8	24	48	72
Ph 800/8 (3 mM)	1.160	5.360	5.750	5.500	3.460	3.200
	± 0.166	± 1.360	± 1.110	± 0.956	± 0.386	± 0.942
Ph 800/1 (0.038 mM)	1.166	5.360	6.170	3.720	2.520	0
	± 0.166	± 1.360	± 1.049	± 0.666	± 0.450	± 0.018
Ph 800/18 (0.068 mM)	0.760	4.140	3.040	0.920	0.080	0
	± 0.114	± 0.671	± 0.457	± 0.616	± 0.052	—

Ph 800/8 was reversible even after 3 days of exposure. Irreversible cell damage was caused by Ph 800/1 only after 72 hr. of treatment and by Ph 800/18 after 24 hr. Thus, Ph 800/18 seemed to be the most toxic, but even this compound required considerable time before a cytotoxic effect was obtained.

Cytostatic effect in vivo. We have previously reported [8] that with the exception of 1:2-dithiolane-3:5-dicarboxylic acid (Ph 800/8) the α -lipoic acid analogues show no cytostatic effect on a hypotetraploid subline of Ehrlich's ascites tumour *in vivo*, and studies of the semicarbazone derivatives pointed to rapid elimination as the cause of these negative results. Less than 1 hr. after the intraperitoneal injection, these drugs were no longer demonstrable in the ascitic fluid nor in the tumour cells. However, the dicarboxylic acid derivative at a dose corresponding to 10% of LD₅₀ given by the intraperitoneal route once daily for five days had a significant inhibitory effect on the rate of multiplication of the hypotetraploid Ehrlich cells *in vivo*.

Similar studies have been carried out with the ELD-tumour. Mice of the DBA/212 strain divided into groups of ten (five of either sex) received 1-5 million tumour cells i.p. For the following six days the animals were given one to three daily intraperitoneal doses as shown in Table 6. The controls were treated with Tyrode's solution, 0.2 ml. per dose. After 9 days the animals were killed by cervical fracture and the ascites tumour cells were withdrawn from the abdominal cavity and counted as described.

Preliminary experiments had shown that the cytostatic effect *in vivo* was greatly depending on the dose of inoculated cells. The experiments

Table 6

The cytostatic effect of Ph 800/8, Ph 800/1 and Ph 800/18 on the ELD-ascites tumour in vivo

Median values with 95% confidence interval [1] are given.

Compound	Single dosage \times no. of i.p. injections in the course of 6 days	Cell number (millions per animal)		Percentage of takes
		Inoculated	After 9 days	
Tyrode's solution	0.2 ml. \times 6	1	280.0 \pm 143.0	50
Ph 800/8	1.0 m-moles/kg. \times 6	1	150.0 \pm 272.0	60
Ph 800/8	1.5 m-moles/kg. \times 6	1	20.0 \pm 0	20
Ph 800/8	2.0 m-moles/kg. \times 6	1	20.0	10
Ph 800/8	3.0 m-moles/kg. \times 6	1	—	0
Tyrode's solution	0.2 ml. \times 15	5	429.0 \pm 119.4	100
Ph 800/8	3.0 m-moles/kg. \times 15	5	223.0 \pm 168.0	100
Ph 800/1	0.038 m-moles/kg. \times 15	5	113.5 \pm 68.8	100
Ph 800/18	0.068 m-moles/kg. \times 15	5	85.0 \pm 65.0	100

summarized in Table 6 showed that at an inoculum dose of one million cells, daily treatment with Ph 800/8 had a cytostatic effect if the individual doses of the compound were not less than 1.5 m-moles per kg., i.e. 1/10 LD₅₀, which is in agreement with our *in vitro* findings.

Doses corresponding to 1/5 LD₅₀ given 15 times in the course of six days to animals which had received five million cells also had a cytostatic effect. This was statistically significant in the case of Ph 800/1 and Ph 800/18, but not in the case of Ph 800/8. Numerous other experiments have confirmed that the cytostatic effect of Ph 800/8 rapidly decreases when the inoculum dose exceeds one million cells.

Therapeutic effect. In order to elucidate the therapeutic effect of the three selected 1:2-dithiolane derivatives a study was made of their influence on the survival time of DBA mice transplanted with 20 million ELD-cells and treated once, twice and three times daily for a total of 5 days. The compounds were administrated by the intraperitoneal route at single doses corresponding to 1/5 LD₅₀. The controls received Tyrode's solution.

From the results presented in Table 7 it appears that none of the three derivatives had any pronounced therapeutic effect. A significant increase in the survival time was only obtained with Ph 800/18 given twice daily, but even in this case the effect was only moderate.

Antimitotic effect in vitro. In order to elucidate the mechanism of the cytostatic effect of the 1:2-dithiolane derivatives a study was made of

Table 7

The therapeutic effect of 1:2-dithiolane derivatives on mice carrying ELD-ascites carcinoma

For survival time, median values with 95% confidence interval [1] are given.

Compound	Single dosage × no. of i.p. injections in the course of 5 days	Percentage of takes	Survival time (days)
Controls	0.2 ml. salt sol. × 5	100	14.0 ± 2.34
Ph 800/8	3.00 m-moles/kg. × 5	100	17.0 ± 2.07
Ph 800/1	0.038 m-moles/kg. × 5	100	14.0 ± 2.30
Ph 800/18	0.068 m-moles/kg. × 5	100	14.0 ± 2.53
Controls	0.2 ml. salt sol. × 10	100	12.0 ± 1.84
Ph 800/8	3.00 m-moles/kg. × 10	100	13.5 ± 1.84
Ph 800/1	0.038 m-moles/kg. × 10	100	12.5 ± 2.30
Ph 800/18	0.068 m-moles/kg. × 10	100	17.0 ± 1.84
Controls	0.2 ml. salt sol. × 15	90	11.0 ± 1.56
Ph 800/8	3.00 m-moles/kg. × 15	90	14.0 ± 2.34
Ph 800/1	0.038 m-moles/kg. × 15	100	13.5 ± 1.15
Ph 800/18	0.068 m-moles/kg. × 15	100	14.0 ± 1.161

Table 8

The effect of 1:2-dithiolane derivatives on the mitotic activity of ELD-tumour cells in vitro

Mitotic coefficient is the no. of mitotic figures per 1000 cells; median values with the 95% confidence interval [1] are given.

Metaphase ratio = $\frac{\text{no. of metaphases}}{\text{total no. of mitoses}} \times 100$; median values with the 95% confidence interval [1] are given.

Treatment	Mitotic coefficient	Metaphase ratio
Controls	35 \pm 12.75	57% \pm 20
Ph 800/8 (3 000 mM)	12 \pm 6.12	63% \pm 34
Ph 800/1 (0.038 mM)	8 \pm 5.10	50% \pm 31
Ph 800/18 (0.068 mM)	12 \pm 6.12	67% \pm 20

their influence on the mitotic activity and the respiratory metabolism of the ELD-cells. From Table 8 it appears that the three selected compounds at a concentration corresponding to 1/5 LD₅₀ had a significant antimitotic effect, but the relative number of metaphases did not change.

Metabolic effect. The metabolic effects of 1:2-dithiolane derivatives

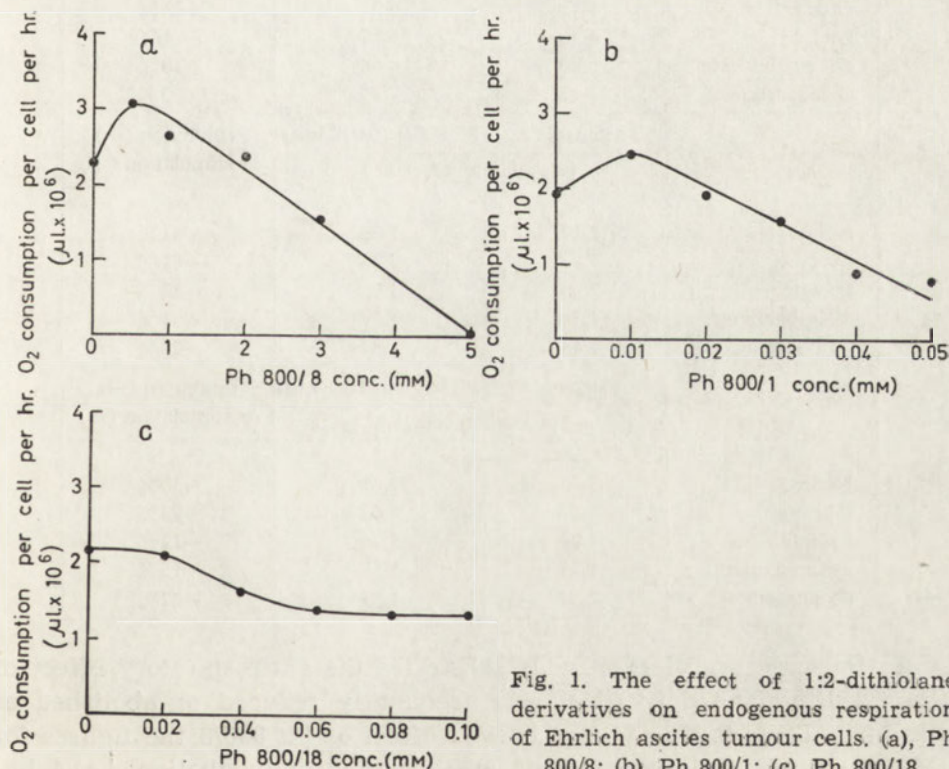


Fig. 1. The effect of 1:2-dithiolane derivatives on endogenous respiration of Ehrlich ascites tumour cells. (a), Ph 800/8; (b), Ph 800/1; (c), Ph 800/18.

have previously been studied in our laboratory [8]. While no antiglycolytic activity could be demonstrated, several of the compounds reduced cellular respiration at a concentration of 0.15 mM. The hypotetraploid Ehrlich ascites tumour cells were more sensitive than the L-fibroblasts but no direct comparison of the cytostatic and the antimetabolic effect could be made at that time.

Figure 1 shows the effect of various concentrations of Ph 800/8, Ph 800/1 and Ph 800/18 on the endogenous respiration of the ELD-cells. Inhibition of cellular respiration was obtained in all cases at concentrations corresponding to $1/5$ LD₅₀. In some experiments, especially in the case of Ph 800/8, this effect was not seen until after 2-3 hr. of incubation.

Table 9

The effect of various substrates on the antirespiratory activity of Ph 800/8, Ph 800/1, and Ph 800/18

Substrate added (5 mM)	Oxygen consumption of ELD-cells		
	Controls	Ph 800/8 (3 mM)	Inhibition (–) or stimulation (+)
	(μl./million cells/4 hr.)		
None	5.12	2.72	–47%
Glucose	5.17	6.24	+21%
Lactate	6.96	6.88	– 1%
α-Ketoglutarate	5.20	4.68	–10%
Oxaloacetate	7.97	7.70	– 3%
	Controls	Ph 800/1 (0.038 mM)	Inhibition (–) or stimulation (+)
	(μl./million cells/1 hr.)		
	None	2.17	1.28
Glucose	1.87	3.38	+81%
Lactate	2.00	1.75	–12%
α-Ketoglutarate	1.78	1.79	+ 1%
Oxaloacetate	2.43	1.84	–24%
	Controls	Ph 800/18 (0.068 mM)	Inhibition (–) or stimulation (+)
	(μl./million cells/1 hr.)		
	None	2.32	1.63
Glucose	2.39	4.62	+93%
Lactate	1.99	1.76	–12%
α-Ketoglutarate	1.85	1.58	–15%
Oxaloacetate	2.59	1.89	–27%

In the presence of exogenous substrates the antirespiratory effect of the 1:2-dithiolane derivatives was frequently reduced or abolished as shown in Table 9. Due to the delayed effect of Ph 800/8 the figures for this compound are given for the whole period of incubation, i.e. 4 hr.,

while the figures for the two other compounds were obtained during the first hour of incubation after the state of equilibrium had been reached. In the presence of glucose the compounds not only produced no respiratory inhibition, but a considerable stimulation of cellular oxygen uptake was usually seen. This effect did not seem to be related to the Crabtree effect, which in these experiments was insignificant.

DISCUSSION

The present experiments have shown that 1:2-dithiolane derivatives may exert a cytostatic effect on the ELD-cells at concentrations corresponding to $1/5$ LD₅₀ or less. However, a cytotoxic effect was only obtained if the cells were exposed to the cytotoxic activity of these compounds for a longer period than the time it takes for the compound to be eliminated from the organism after intraperitoneal injection [8]. Permanent infusion or perfusion would seem to be the only way of maintaining an active serum level for a sufficiently long time. However, the relatively low rate of solubility of the majority of the 1:2-dithiolane derivatives so far synthesized makes them less suitable for such methods of administration. Therefore, the possibility of sensitizing the tumour cells to the cytotoxic effect of these compounds should be considered.

The mechanism of the cytostatic effect has not yet been elucidated. Physico-chemical investigations of Schotte & Nygård [15] as well as biological studies of Machlis [11] point to the five-membered disulphide ring as the active component of these compounds. Sulphydryl-disulphide interchange is known to be involved in many protein reactions such as denaturation, gel formation and clotting [see 7]. Gelation of protein through intermolecular disulphide bond formation probably plays an important part in the construction of the mitotic apparatus [see 12, 13], and it is, therefore, conceivable that the growth inhibitory effect of 1:2-dithiolane derivatives is due to interference with this process. Di Carlo [2] has demonstrated a clear antimitotic effect of α -lipoic acid on onion roots, and a similar effect of other 1:2-dithiolane derivatives on the ELD-cells was found in the present investigation. However, the relative number of metaphases did not change, as one would have expected in case of damage to the mitotic spindle. These results indicate that the process of cell division is inhibited at an early phase, as if the cytostatic effect of α -lipoic acid analogues were due to interference with cellular metabolism.

The antirespiratory effect of the α -lipoic acid analogues pointed to α -lipoic acid antagonism, as an explanation of their cytostatic effects. However, α -lipoic acid also had a growth inhibiting effect, and numerous attempts to neutralize the influence of the analogues on cell growth

or cell metabolism by adding this coenzyme to the medium were unsuccessful.

Henderson & Eakin [6] have shown an inhibitory effect of cyclic disulphides on several dehydrogenases, malic acid dehydrogenase being the most sensitive. If the present derivatives had a similar effect, one would expect oxaloacetate, but not α -ketoglutarate to be able to neutralize their antimetabolic activity. However, this was not the case. On the contrary, α -ketoglutarate seemed more efficient than oxaloacetate in neutralizing the antirespiratory effect of Ph 800/1 and Ph 800/18.

The metabolic effects of the 1:2-dithiolane derivatives resemble those obtained by exposing ELD-cells to high and low oxygen tensions, which points to fat metabolism as the site of their action [9, 14]. However, further experiments are needed before any final conclusions can be drawn.

The results of the present metabolic studies suggest that the sensitivity of the tumour cells to the 1:2-dithiolane derivatives may be increased if the cells are prevented from utilizing exogenous substrates. This points to the combination with antiglycolytic agents as a way of increasing the cytotoxic and thereby also the therapeutic effect of 1:2-dithiolane derivatives. However, this concept does not necessarily mean that the cytostatic effect observed in the presence of exogenous substrates is related to the influence on endogenous respiration. Thus, the final evaluation of the antineoplastic potentialities of the 1:2-dithiolane derivatives requires further studies of the biochemical mechanism of their cytostatic effects.

This investigation has been supported by grants to the Fibiger Laboratory from The Danish National Research Foundation, King Christian X's Memorial Fund and Arvid Nilssons Foundation.

SUMMARY

The effect of α -lipoic acid analogues on the growth and metabolism of Ehrlich ascites tumour cells was studied. 1:2-Dithiolane derivatives inhibited the growth of the cells *in vitro* and *in vivo*. Several of the compounds at concentration corresponding to $1/5$ LD₅₀ inhibited endogenous respiration of the cells. In the presence of the exogenous substrates the antirespiratory effect was reduced or abolished.

REFERENCES

- [1] Dean R. B. & Dixon W. J. - *Analyt. Chem.* **23**, 636, 1951.
- [2] Carlo V. di - *Arzneimittel-Forsch.* **7**, 706, 1957.
- [3] Carlo V. di, Giordano R. & Giordano G. G. - *Atti Soc. Lombarda Sci. Med. Biol.* **13**, 447, 1958.

- [4] Carlo V. di, Maimone G. & Giordano G. - *Naturwiss.* **45**, 44, 1958.
- [5] Eagle H. - *Science* **130**, 432, 1959.
- [6] Henderson R. F. & Eakin R. E. - *Biochem. Biophys. Res. Comm.* **3**, 169, 1960.
- [7] Jensen E. V. - *Science* **130**, 1319, 1959.
- [8] Kieler J. - *Biochem. Pharmacol.* **11**, 453, 1962.
- [9] Kieler J., Nissen N. I. & Bicz W. - *Acta U.I.C.C.* **18**, 228, 1962.
- [10] Lettré H. - *Naturwiss.* **45**, 217, 1958.
- [11] Machlis L. - *Arch. Biochem. Biophys.* **70**, 413, 1957.
- [12] Mazia D., *Glutathione* (S. Colowick, ed.) p. 209, Academic Press, New York, 1954.
- [13] Mazia D. - *Symposia Soc. Exptl. Biol.* **9**, 335, 1955.
- [14] Nissen N. I., *Undersøgelser over vækst og stofskifteprocesser hos Ehrlich ascites tumor celler*. Thesis, Copenhagen 1962.
- [15] Schotte L. & Nygård B. - *Biochem. Pharmacol.* **11**, 445, 1962.

POCHODNE 1,2-DWUTIOCYKLOPENTANU

III. ANTINEOPLASTYCZNA AKTYWNOŚĆ

Streszczenie

Przebadano wpływ pochodnych kwasu α -liponowego na wzrost i metabolizm komórek wysiękowego raka Ehrlicha. Stwierdzono, że niektóre z tych związków hamują wzrost komórek Ehrlicha *in vitro* i *in vivo*. W stężeniu odpowiadającym $1/5 LD_{50}$ hamują one endogenne oddychanie. W obecności substratów egzogennych hamowanie oddychania ulega zmniejszeniu lub zanika.

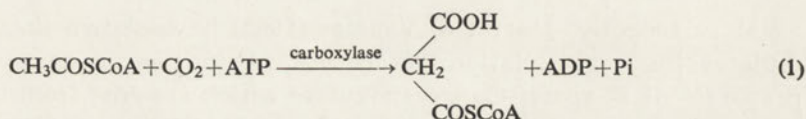
Received 30 December 1963.

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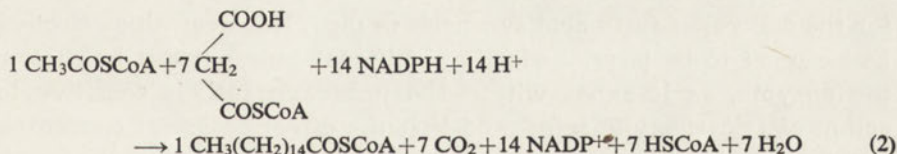
ON THE POSSIBLE ROLE OF TRICARBOXYLIC ACIDS IN THE CONTROL OF FATTY ACID SYNTHESIS

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In animal tissue, the first step in the formation of long-chain fatty acids from acetyl-CoA is the carboxylation of acetyl-CoA to form malonyl-CoA (Wakil, 1961). This reaction requires ATP and CO₂, and is catalysed by acetyl-CoA carboxylase, a biotin-containing enzyme:



In the subsequent steps, units of malonyl-CoA condense successively with a single priming unit of acetyl-CoA. During the addition of each molecule of malonyl-CoA, one molecule of CO₂ is lost, resulting in the formation of a new β -keto acid by the net addition of two carbon atoms. According to the current view, the β -keto acid is then reduced to the fully saturated acid by NADPH¹. The overall reaction for the formation of palmityl-CoA from acetyl-CoA and malonyl-CoA may thus be written:



Before the discovery of the malonyl-CoA pathway for the synthesis of fatty acids, it had already been shown that citrate and isocitrate stimulate fatty acid synthesis from acetate in purified cell-free systems. This effect has been observed in preparations from pigeon-liver (Brady

¹ Abbreviations: NADP⁺, NADPH, oxidized and reduced nicotine adenine dinucleotide phosphate; NADH, reduced nicotine adenine dinucleotide; DNP, 2,4-dinitrophenol. The 1-C atom of citrate refers to the C atom derived from the 1-C of acetate when citrate is formed enzymically from acetate and oxaloacetate.

& Gurin, 1952), lactating mammary gland (Popják & Tietz, 1955) and adipose tissue (Martin & Vagelos, 1962). Under certain conditions, stimulation may reach 60-fold, and in at least one system, the mammary gland preparation described by Abraham, Matthes & Chaikoff (1961), fatty acid synthesis from acetate does not take place unless citrate is added.

Several suggestions have been put forward to explain the stimulatory effect of tricarboxylic acids. At one time it was thought to be due to an increase in the supply of NADPH generated by the oxidation of isocitrate (Brady, Mamoon & Stadtman, 1956). However, this is no longer considered likely, since the rate-limiting step in fatty acid synthesis is reaction (1), which does not require NADPH. Hülsmann (1960) has suggested that tricarboxylic acids stimulate fatty acid synthesis by forming oxalosuccinate, which then acts as a donor in a transcarboxylation resulting in the formation of malonyl-CoA from acetyl-CoA. This, however, has been disproved by Numa, Matsushashi & Lynen (1961), who showed that oxalosuccinate cannot replace citrate in a fatty-acid synthesising system from rat liver.

More recently, Martin & Vagelos (1962) have shown that citrate stimulates the carboxylation of acetyl-CoA in the presence of purified preparations of acetyl-CoA carboxylase which are free from the enzymes necessary for coupling the reduction of NADP^+ to NADPH with the oxidation of citrate. Vagelos, Alberts & Martin (1963) have now shown that this effect of citrate is due to activation of the carboxylase, possibly by altering its state of molecular aggregation. Both D- and L-isocitrate have also been shown to have a stimulatory effect on acetyl-CoA carboxylase about equal to that of citrate (Lynen, Matsushashi, Numa & Schweitzer, 1963).

The activation of acetyl-CoA carboxylase by tricarboxylic acids has aroused a good deal of interest because it suggests a possible mechanism for the control of fatty acid synthesis *in vivo*. However, this effect cannot be assumed to be physiologically significant unless it can be shown that the enzyme, as it exists within the unbroken cell, is sensitive to the action of tricarboxylic acids, and that the effect occurs at concentrations within the physiological range.

We have recently described a rat liver homogenate (Iliffe & Myant, 1964) which resembles the unbroken cell more closely, in some respects, than the cell-free, or particle-free, preparations used by most other workers for studying fatty acid synthesis. In our preparation ("whole homogenate"), the cell sap and all the particles except the nuclei are retained, and no cofactors are added from outside. All the cofactors required for fatty acid synthesis are generated from endogenous sources within the homogenate itself; the ATP is generated by phosphorylating

mitochondria, the NADPH by oxidation of endogenous substrates and the CO_2 by mitochondrial oxidation of succinate.

We have shown that tricarboxylic acids have little or no stimulatory effect upon the incorporation of $[1-^{14}\text{C}]$ acetate into fatty acids in the whole homogenate (Myant & Iliffe, 1963). We suggested that this might be due to the formation of non-radioactive acetyl-CoA from citrate by the action of the citrate cleavage enzyme (Srere, 1959). We pointed out, however, that our results raised the possibility that acetyl-CoA carboxylase is not sensitive to the action of tricarboxylic acids within the unbroken cell or whole homogenate, and that it only becomes so during certain stages in the preparation of particle-free systems, or of the purified enzyme itself. It was clearly important to establish whether or not this is so.

In this paper we describe further attempts to demonstrate a stimulatory effect of tricarboxylic acids on the carboxylation reaction in the whole homogenate. We have been unable to find more than a trivial stimulatory effect on $[1-^{14}\text{C}]$ acetate incorporation into fatty acids. However, our experiments show that the carboxylation reaction can be stimulated by citrate, though only at concentrations that are above the physiological range.

RESULTS

Incorporation of $[1-^{14}\text{C}]$ acetate into fatty acids in the whole homogenate

In the presence of optimal concentrations of acetate, suspensions of the whole homogenate, containing the equivalent of 400 mg. of wet liver, incorporated between 100 and 300 μmoles of $[1-^{14}\text{C}]$ acetate into long-chain fatty acids during a 3-hr. incubation (Table 1). This rate of incorporation is about the same as that shown by a similar weight of rat liver slices (Chernick & Chaikoff, 1951).

In the whole homogenate, synthesis of fatty acids from acetate is probably limited by the supply of ATP, since the incorporation of $[1-^{14}\text{C}]$ acetate was diminished by factors which interfere with oxidative phosphorylation, and was increased by stimulating the production of endogenous ATP or by adding exogenous ATP. As shown in Table 1, in the presence of normally phosphorylating mitochondria, the concentration of endogenous ATP was maintained at or above its initial level throughout the incubation. When phosphorylation was inhibited by DNP, ATP disappeared rapidly from the incubation mixture and the incorporation of acetate into fatty acids was markedly diminished. If, on the other hand, the ATP level was raised by adding ATP or by stimulating phosphorylation with NADH, incorporation was increased.

The influence of citrate and isocitrate upon [1-¹⁴C]acetate incorporation into fatty acids in the whole homogenate

Since the incorporation of [1-¹⁴C]acetate in the whole homogenate was influenced by the ATP level, it was clear that tricarboxylic acids might affect incorporation indirectly by stimulating, or interfering with, the supply of ATP from the mitochondria. Accordingly, in all subsequent experiments with whole homogenates, the ATP level was measured at the end of the incubation.

In the standard incubation mixture (Table 1), the energy for oxidative phosphorylation is supplied by 15 mM-succinate. When the succinate was replaced by citrate or isocitrate, there was a slight or moderate increase in [1-¹⁴C]acetate incorporation over most of the range of concentration tested, though with citrate concentrations above 15 mM, incorporation was diminished (Table 2). When there was an increase in ¹⁴C incorporation this was not accompanied by a corresponding increase in the ATP level at the end of the incubation. On the contrary, the ATP level tended to be lower with citrate or isocitrate than with succinate alone.

In the presence of the standard 15 mM-succinate, citrate added at a concentration of 5 mM had a slight stimulatory effect on the incorpo-

Table 1

Influence of the supply of ATP upon the incorporation of [1-¹⁴C]acetate into fatty acids in the whole homogenate

Each flask contained 2.0 ml. of homogenate from 400 mg. wet liver in a final volume of 4.5 ml., with tris-HCl buffer (45 mM, pH 7.4), KCl (40 mM), MgCl₂ (4 mM), K acetate (2.5 mM), Na [1-¹⁴C]acetate (0.01 mM), K₂HPO₄ (1.0 mM), reduced glutathione (5.0 mM) and K succinate (15 mM). The flasks were incubated in duplicate, with shaking, for 3 hr. at 37° with air as the gas phase.

The concentration of ATP in the incubation mixture was measured at the beginning and end of the incubation (Fletcher & Myant, 1961). The amount of acetate incorporated into fatty acids was calculated from the specific activity of the added [1-¹⁴C]acetate and the activity recovered in the fatty acids, no allowance being made for endogenous acetate.

Experiment	Additions (mM)	ATP concentration (mM)		Acetate incorporated (μmoles)
		Initial	Final	
1	None	0.24	0.28	207
	DNP (0.1)	0.22*	0	25
	ATP (2.0)	2.20	1.14	1560
2	None	0.22*	0.39	292
	NADH (0.5)	0.22*	0.77	600

* Average values taken from other experiments.

Table 2

Effect of replacing succinate by citrate or isocitrate upon [1-¹⁴C]acetate incorporation into fatty acids in the whole homogenate

The conditions of incubation were the same as in Table 1, except that succinate was replaced by citrate or DL-isocitrate where shown in the Table.

Additions (mm)	¹⁴ C incorporated	Final ATP concentration
Succinate (15)	100	100
Citrate (10)	149	51
(15)	118	101
(20)	73	74
Isocitrate (10)	98	47
(15)	165	76
(20)	165	66

ration of [1-¹⁴C]acetate, but higher concentrations either had no effect or were inhibitory (Table 3). Isocitrate added at low concentrations in the presence of succinate stimulated incorporation, but was inhibitory at concentrations above 10 mM (Table 3). When citrate or isocitrate were added in the presence of succinate, the level of ATP at the end of the

Table 3

Effect of citrate or isocitrate, in addition to succinate, upon [1-¹⁴C]acetate incorporation into fatty acids in the whole homogenate

The conditions of incubation were the same as in Table 1 (all flasks containing 15 mM succinate), except for the additions of citrate, DL-isocitrate, or ATP shown in the Table.

Additions (mm)	¹⁴ C incorporated	Final ATP concentration
None	100	100
Citrate (5)	127	72
(15)	44	67
Isocitrate (5)	192	37
(10)	113	43
(15)	81	—
(20)	37	39
(3 × 22.5)*	23	—
ATP (2)	472	1670
ATP (2) + isocitrate (20)	314	1440

* 22.5 mM-DL-isocitrate added at 0, 1 and 2 hr.; in these flasks, the acetate concentration was 10 mM.

incubation was usually lower than with succinate alone. This raised the possibility that a stimulatory effect on the carboxylation reaction was nullified by the fall in the ATP level. However, this seems unlikely, because isocitrate had no stimulatory effect in the presence of optimal concentrations of added ATP (Table 3).

These results showed that the incorporation of $[1-^{14}\text{C}]$ acetate into fatty acids in the whole homogenate could be stimulated by tricarboxylic acids and that the effect was not mediated by a change in the level of ATP. However, stimulation occurred only in rather restricted conditions (Table 2 and 3) and, when it did occur, was much smaller than that observed by other workers using more highly fractionated systems or purified enzyme preparations.

We considered the possibility that the absence of a more marked stimulatory effect might be due to rapid removal of the added tricarboxylic acid by mitochondrial oxidation or by some other metabolic pathway. We therefore tested the effect of adding isocitrate in three

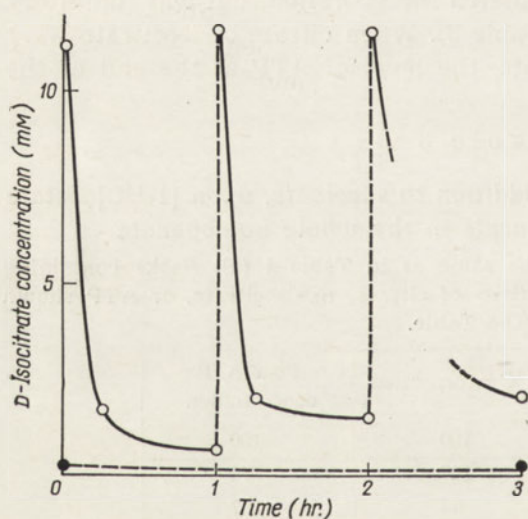
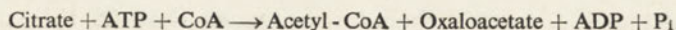


Fig. 1. Concentration of D-isocitrate in whole homogenate with three additions of 22.5 mM-DL-isocitrate at 0, 1 and 2 hr. (○) and in control flasks with no added isocitrate (●). Conditions of incubation and composition of incubation mixture as in Table 1. D-Isocitrate assayed by a modification of the method of Stern (1957).

successive doses. At the same time, we measured the concentration of isocitrate during the incubation. Three doses of 22.5 mM-isocitrate inhibited $[1-^{14}\text{C}]$ acetate incorporation into fatty acids (Table 3). In the control flasks, the concentration of endogenous isocitrate was less than 0.1 mM at the beginning of the incubation and fell to zero by the end of the incubation; in the flasks to which isocitrate was added, the concentration fell rapidly after each addition, but nevertheless remained several times higher than the level in the control flasks during much of the incubation (Fig. 1).

*Incorporation of [1,5-¹⁴C]citrate into fatty acids
in the whole homogenate*

Spencer & Lowenstein (1962) have shown that the 1-C atom of citrate is incorporated into fatty acids in tissue preparations which contain the citrate cleavage enzyme (Srere, 1959). This enzyme generates acetyl-CoA from the 1- and 2-position carbon atoms of citrate in the presence of ATP and CoA:



Since the incorporation of [1,5-¹⁴C]citrate into fatty acids is almost completely inhibited by avidin (Spencer & Lowenstein, 1962), an anti-biotin factor, nearly all the 1-position C of citrate that enters fatty acids must do so *via* acetyl-CoA and the malonyl-CoA pathway.

It therefore seemed possible that in the whole homogenate, which must contain the citrate cleavage enzyme, the added citrate might give rise to enough non-radioactive acetyl-CoA to diminish the incorporation of radioactive acetate into fatty acids. This would tend to mask a stimulatory effect of citrate on the carboxylation reaction. Isocitrate, in so far as it is converted to citrate, could also give rise to acetyl-CoA.

Table 4

*Incorporation of 1-position C atom from [1-¹⁴C]acetate and [1,5-¹⁴C]citrate
into fatty acids in the whole homogenate*

The conditions of incubation and the contents of the flasks were the same as in Table 1, except that succinate was omitted in the flasks containing added citrate. Since the incorporation of the 5-C atom of citrate into fatty acids is only about 1/20 that of the 1-C atom (Spencer & Lowenstein, 1962), incorporation of 1-position ¹⁴C from citrate was calculated from the radioactivity recovered in the fatty acids, and the specific activity of the [1,5-¹⁴C]citrate divided by two. Note that in this experiment, the ATP level was slightly higher in the presence of citrate than in its absence. However, the difference was not great enough to account for the fact that the incorporation of citrate ¹⁴C was five times as great as that of acetate ¹⁴C.

Substrate (mm)	Incorporation of 1-position ¹⁴ C (μatoms)	Final ATP concentration (mm)
[1- ¹⁴ C]Acetate (5) + succinate (15)	102	0.14
[1- ¹⁴ C]Acetate (5) + citrate (10)	117	0.17
[1,5- ¹⁴ C]Citrate (10) + acetate (5)	552	0.20

That citrate does, in fact, contribute to the acetyl-CoA pool in the whole homogenate was shown by experiments in which we measured the incorporation of [1,5-¹⁴C]citrate into fatty acids. In the experiment shown in Table 4, 10 mm-citrate had a slight, though scarcely significant,

stimulatory effect on the incorporation of $[1-^{14}\text{C}]$ acetate. But at this concentration, the 1-C atom of citrate was incorporated about five times as efficiently as the carbon atoms of 5 mM-acetate. Thus, in the flasks containing 5 mM- $[1-^{14}\text{C}]$ acetate and 10 mM-citrate, about 5% of the acetyl-CoA converted to fatty acids was derived from citrate. In the absence of a corresponding increase in the maximal capacity of the carboxylation reaction, this should have diminished the incorporation of $[1-^{14}\text{C}]$ acetate, compared with that incorporated in the flasks containing $[1-^{14}\text{C}]$ acetate but no citrate. The results therefore suggest that in this preparation the carboxylation reaction was stimulated by citrate.

We were able to obtain more evidence for a stimulatory effect by comparing the incorporation of $[1-^{14}\text{C}]$ acetate in the absence of citrate with that of the 1-C atom of $[1,5-^{14}\text{C}]$ citrate, at increasing substrate concentrations (Fig. 2).

The reasoning behind these experiments was as follows. The maximal incorporation of $[1-^{14}\text{C}]$ acetate provides a measure of the capacity of the carboxylation step in the absence of citrate, since this is the rate-limiting step in fatty acid synthesis from acetate in the whole homogenate (Iliffe & Myant, 1964). The rate-limiting step in fatty acid synthesis from

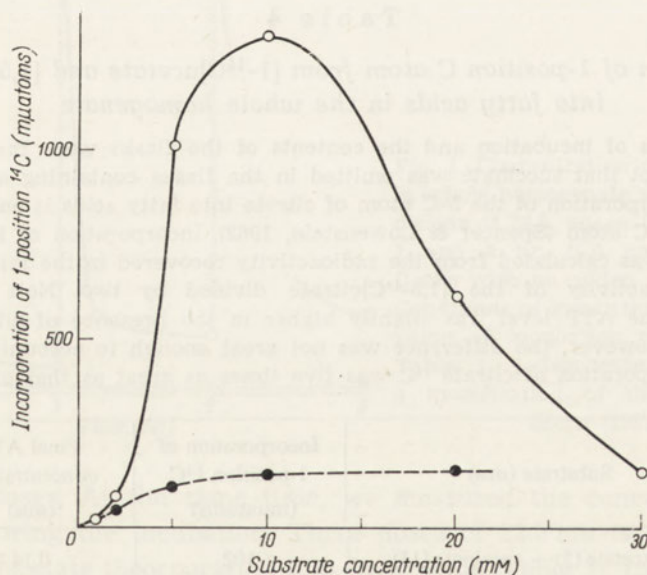


Fig. 2. Incorporation of 1-position C atom of $[1,5-^{14}\text{C}]$ citrate (O) and of $[1-^{14}\text{C}]$ acetate (●) into fatty acids at increasing substrate concentrations. Paired flasks containing whole homogenate from the pooled livers of two rats were incubated as in Table 1. The contents of the flasks were the same as in Table 1, except that the succinate concentration was reduced from 15 mM to 10 mM and the concentration of acetate was as shown in the Figure. Incorporation of 1-position ^{14}C from citrate was calculated as in Table 4.

citrate in our preparation is either the formation of acetyl-CoA from citrate by the action of the cleavage enzyme, or the carboxylation of acetyl-CoA. Whichever is the case, the capacity of the carboxylation step in the presence of citrate cannot be less than the maximal incorporation of the 1-C atom of citrate into fatty acids.

In the experiment shown in Fig. 2, incorporation of the 1-C atom of citrate reached a maximum of 1320 μ atoms at a concentration of 10 mM, whereas the maximal incorporation of acetate in the absence of citrate was only 160 μ atoms. The capacity of the carboxylation step must, therefore, have been increased at least 8-fold by 10 mM-citrate. The fall in ^{14}C incorporation at high citrate concentrations was probably due to depression of the ATP level, which always occurred at concentrations of citrate above 15 mM. The sigmoid shape of the ascending part of the citrate curve is to be expected if citrate not only provides acetyl-CoA units for fatty acid synthesis, but also stimulates the carboxylation of acetyl-CoA.

DISCUSSION

Many hypotheses have been put forward to explain how fatty acid synthesis is controlled in the living animal. At one time or another it has been suggested that control is exerted by changes in the supply of NADPH (Langdon, 1957) or of ATP (Fletcher & Myant, 1960), by changes in the concentration of malonyl-CoA synthetase (Gibson & Hubbard, 1960) or in the concentration (Numa, Matsushashi & Lynen, 1961) or degree of activation (Vagelos, Alberts & Martin, 1963) of acetyl-CoA carboxylase; and Lynen, Matsushashi, Numa & Schweizer (1963) have now suggested that CoA derivatives of long-chain fatty acids exert a controlling influence by inhibiting acetyl-CoA carboxylase.

It is, of course, possible that fatty acid synthesis is regulated by more than one mechanism, and that the factor which exerts the greatest influence at any given moment varies according to conditions inside the cell. But before any of these mechanisms can be assumed to be of physiological significance, it must be studied in systems of increasing complexity and, ultimately, in the whole cell.

Our experiments show some of the difficulties that arise with a system as complex as the whole homogenate. Although citrate, and possibly isocitrate, stimulate the carboxylation step in the whole homogenate, their effect on $[1-^{14}\text{C}]$ acetate incorporation into fatty acids is negligible by comparison with the stimulatory effect observed in more highly fractionated systems. In the case of citrate, this appears to be due, at least partly, to two factors. First, the presence of the citrate cleavage enzyme results in a large increase in the amount of acetyl-CoA when citrate is added to the preparation; the amount of $[1-^{14}\text{C}]$ acetate converted to malonyl-CoA is, therefore, less than it would otherwise be.

Secondly, some of the added citrate is oxidized to CO_2 , as shown by the evolution of $^{14}\text{CO}_2$ from $[1,5\text{-}^{14}\text{C}]$ citrate incubated in the whole homogenate (Iliffe & Myant, unpublished work). It is possible that isocitrate also forms acetyl-CoA in our preparation, though we have no evidence for this. What appears to be a more important factor in diminishing the stimulatory effect of isocitrate is its relatively rapid rate of metabolism in the whole homogenate (Fig. 1).

A further complication in these experiments arises from the fact that $[1\text{-}^{14}\text{C}]$ acetate incorporation in the whole homogenate is dependent upon ATP generated by mitochondrial phosphorylation. Tricarboxylic acids at high concentrations depress the level of ATP and thus diminish $[1\text{-}^{14}\text{C}]$ acetate incorporation into fatty acids.

There remains the question of the physiological significance of the activating effect of citrate. Our earlier suggestion (Myant & Iliffe, 1963) that activation of acetyl-CoA carboxylase is an artifact may not be necessary. Nevertheless, the concentration of citrate required to activate acetyl-CoA carboxylase is much higher than the concentration in normal liver. Activation of purified enzyme preparations does not begin until the citrate concentration is raised above 2 mM (Vagelos, Alberts & Martin, 1963). The position of the inflection in the $[1,5\text{-}^{14}\text{C}]$ citrate curve (Fig. 2) also suggests that activation in the whole homogenate does not begin until the concentration exceeds 1 mM. In normal liver, however, the concentration of citrate is less than 0.2 mM (Frohman, Orten & Smith, 1951). It seems unlikely, therefore, that citrate activation plays more than a subsidiary role in the regulation of fatty acid synthesis in the whole cell, unless the enzyme is sensitive to lower concentrations of citrate within the whole cell than in subcellular fractions of liver.

SUMMARY

Citrate and isocitrate, both of which activate purified preparations of acetyl-CoA carboxylase, have no effect on the incorporation of $[1\text{-}^{14}\text{C}]$ acetate into fatty acids in a whole homogenate of rat liver. But the incorporation of the 1-position carbon atom of $[1,5\text{-}^{14}\text{C}]$ citrate added at increasing substrate concentrations shows that the carboxylation step is stimulated by citrate in the whole homogenate. The reasons for this anomaly are discussed. It is pointed out that the concentration of citrate required to activate acetyl-CoA carboxylase is several times higher than the concentration of citrate in normal liver.

REFERENCES

- [1] Abraham S., Matthes K. J. & Chaikoff I. L. - *Biochim. Biophys. Acta* **49**, 268, 1961.
- [2] Brady R. O. & Gurin S. - *J. biol. Chem.* **199**, 421, 1952.
- [3] Brady R. O., Mamoon A. M. & Stadtman E. R. - *J. biol. Chem.* **222**, 795, 1956.
- [4] Chernick S. S. & Chaikoff I. L. - *J. biol. Chem.* **188**, 389, 1951.
- [5] Fletcher K. & Myant N. B. - *J. Physiol.* **154**, 145, 1960.
- [6] Fletcher K. & Myant N. B. - *J. Physiol.* **157**, 542, 1961.
- [7] Frohman C. E., Orten J. M. & Smith A. H. - *J. biol. Chem.* **193**, 803, 1951.
- [8] Gibson D. M. & Hubbard D. D. - *Biochem. biophys. Res. Commun.* **3**, 531, 1960.
- [9] Hülsmann W. C. - *Biochim. biophys. Acta* **45**, 623, 1960.
- [10] Iliffe J. & Myant N. B. - *Biochem. J.* **91**, 369, 1964.
- [11] Langdon R. G. - *J. biol. Chem.* **226**, 615, 1957.
- [12] Lynen F., Matsushashi M., Numa S. & Schweizer E., in *The Control of Lipid Metabolism, Biochemical Society Symposium No. 24 held at Oxford, 19 July, 1963* (J. K. Grant, ed.) pp. 43-56. Academic Press, London 1963.
- [13] Martin D. B. & Vagelos P. R. - *J. biol. Chem.* **237**, 1787, 1962.
- [14] Myant N. B. & Iliffe J., in *The Control of Lipid Metabolism, Biochemical Society Symposium No. 24 held at Oxford, 19 July, 1963* (J. K. Grant, ed.) pp. 145-154. Academic Press, London 1963.
- [15] Numa S., Matsushashi M. & Lynen F. - *Biochem. Z.*, **334**, 203, 1961.
- [16] Popják G. & Tietz A. - *Biochem. J.* **60**, 147, 1955.
- [17] Spencer A. F. & Lowenstein J. M. - *J. biol. Chem.* **237**, 3640, 1962.
- [18] Srere P. A. - *J. biol. Chem.* **234**, 2544, 1959.
- [19] Stern J. R., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) Vol. III, pp. 425-431. Academic Press, New York 1957.
- [20] Vagelos P. R., Alberts A. W. & Martin D. B. - *J. biol. Chem.* **238**, 533, 1963.
- [21] Wakil S. J. - *J. Lipid. Res.* **2**, 1, 1961.

O PRZYPUSZCZALNEJ ROLI KWASÓW TRÓJKARBOKSYLOWYCH
W KONTROLI SYNTEZY KWASÓW TŁUSZCZOWYCH

Streszczenie

Zarówno cytrynian jak i izocytrynian, które aktywują oczyszczone preparaty acetylo-CoA karboksylazy, są bez wpływu na włączanie $[1-^{14}\text{C}]$ octanu do kwasów tłuszczowych w pełnym homogenacie wątroby szczura. Natomiast inkorporacja C_1 z $[1,5-^{14}\text{C}]$ cytrynianu dodawanego we wzrastających ilościach wskazuje na aktywację karboksylacji przez cytrynian. Przedyskutowano przyczyny tej rozbieżności. Zwrócono uwagę, że stężenie cytrynianu konieczne do aktywacji acetylo-CoA karboksylazy jest kilkakrotnie wyższe niż normalne stężenie cytrynianu w wątrobie.

Received 30 December 1963.

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ISOLATION OF NON-HAEMOGLOBIN PROTEINS OF THE RED BLOOD CELL BY THE USE OF RIVANOL

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Recently, Rivanol (6,9-diamino-2-ethoxyacridine lactate) has been widely used for protein analysis. It was successfully applied for isolation of the virus of the tobacco mosaic disease [5], serum γ -globulin [10, 11], transferrin [1] and ceruloplasmin [19] as well as for separation of serum proteins [13]. It was also shown that Rivanol forms complexes with nucleic acids [2] and precipitates certain mucopolysaccharides [21].

The purpose of the present work was to elaborate a method for isolation and fractionation of non-haemoglobin proteins of the red blood cells by means of Rivanol. For comparison, non-haemoglobin proteins were isolated using DEAE-cellulose according to Hennesey & Haffner [8, 9].

MATERIALS AND METHODS

For experiments, preserved human blood obtained from the Institute of Haematology, Warszawa, was used. In each blood sample haematocrite reading was made and a smear was stained according to Giemza-May-Grünwald. Only the blood which in these tests proved to be normal and without any traces of haemolysis, was used.

Haemolysate. Approx. 200 ml. of blood was centrifuged at 4° for 20 min. at 9000 r.p.m. on a MSE HS-17 centrifuge. The cells were washed four times with a cold 0.9% NaCl solution, the washings being discarded together with the thin layer of leucocytes. The last washings gave no reaction with sulphosalicylic acid. The packed red blood cells were frozen and thawed three times, then added with an equal volume of distilled water to complete haemolysis.

Isolation of the non-haemoglobin proteins by the Hennesey & Haffner method [8]. The haemolysate was mixed with a suspension of DEAE-cellulose and centrifuged. Nearly all the haemoglobin was present in the supernatant; only minute amounts were adsorbed on the DEAE-cellulose

and these were removed by washing with a 3 mM-phosphate buffer, pH 7. From the DEAE-cellulose the adsorbed non-haemoglobin proteins were eluted with 0.5 M-KCl solution during 1 hr. at 4°, with constant stirring. After centrifugation the supernatant was dialysed against distilled water for three days at 4°, until chloride-free and the dialysis residue was lyophilized. A white or pinkish powder was obtained.

Isolation of non-haemoglobin proteins using Rivanol precipitation. To the haemolysate a 5-fold volume of 0.3% Rivanol was added. The precipitate was centrifuged and the supernatant containing both haemoglobin and a part of the Rivanol was discarded. To remove the remaining haemoglobin, the precipitate was mixed thoroughly in Sorvall homogenizer with a small amount of 0.3% Rivanol and then washed 5 times with the Rivanol solution added in quantities equal to the initial volume of the haemolysate. The Rivanol-protein precipitate was extracted with 0.5 M-KCl for 24 hr. at 4°. The yellow extract was dialysed against distilled water for 3-4 days at 4° until colourless and chloride-ion-free, and then lyophilized. A white or slightly pink power was obtained.

The degree of purification of the preparations was estimated by measuring the protein and iron contents. The protein was determined by the Folin & Ciocalteu method [3], and iron according to Ramsay [16].

Starch gel electrophoresis. The method of Haut *et al.* [6, 7] was employed for electrophoretic separation and for staining of the obtained fractions. For one separation, 10 ml. of 5% protein solution in tris buffer, pH 9.5, was used. After separation the starch block was cut into 3 horizontal layers: one was stained with Amido Black, the second with a benzidine reagent to locate haemine; and the third one was immersed in a 3% H₂O₂ solution and the areas where gas bubbled up were marked to locate catalase activity.

RESULTS

The lyophilized preparation obtained by Rivanol precipitation contained 40.8% protein; the protein to iron ratio was 4100. The preparation obtained by the Hennesey & Haffner method contained 43.2% protein and the protein to iron ratio was 5040. In the haemolysate this ratio was 300 and in the serum 70 000.

The diagrams of starch gel electrophoresis are presented in Fig. 1. The whole haemolysate was separated into six fractions: two haemoglobin ones, A₁ and A₂, and four non-haemoglobin fractions marked I-IV. The number of fractions, intensity of staining with Amido Black, width of the individual zones, benzidine test, and position of catalase activity (fraction III) were in agreement with those reported by Haut *et al.* [6, 7].

The preparation obtained using Rivanol was separated into thirteen fractions. The first three corresponded to fractions I, II and III of the haemolysate. Fractions 6, 8 and 11 showed the same mobility as fractions IV, A₂ and A₁, respectively. Fractions 4, 5, 7, 9, and 10 were located

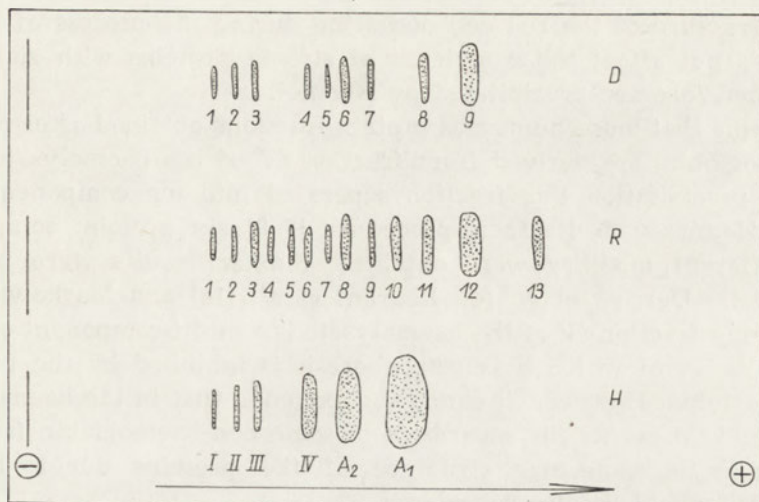


Fig. 1. Electrophoresis on starch gel of red blood cell preparations. (H), Haemolysate; (R), non-haemoglobin protein isolated by Rivanol precipitation, and (D), isolated by the Hennesey & Haffner DEAE-cellulose procedure. Amido Black staining.

between the haemolysate fractions, and fractions 12 and 13 were beyond fraction A₁. None of the fractions gave benzidine-positive reaction, and catalase activity was present in fraction 3.

The preparation obtained by the Hennesey & Haffner procedure separated into nine fractions only. It can be seen in the Figure that from this preparation fractions 4, 5, 10, 12 and 13 of our Rivanol preparation, were absent. None of the obtained fractions gave a positive benzidine test, and no catalase activity was found.

DISCUSSION

In neutral medium Rivanol forms insoluble complexes with those proteins the isoelectric point of which is lower than 5.2 [12, 17, 18, 20]. Thus serum albumin, α -globulin and some β -globulin are precipitated. The isoelectric point of haemoglobin and the non-haemoglobin protein isolated by Haut *et al.* [6, 7] is higher but only haemoglobin is not precipitated by Rivanol.

On electrophoresis the red blood cells appeared to have acidic character corresponding to isoelectric point 4. It could be anticipated

that Rivanol by neutralizing the negative charge would cause their aggregation, and indeed we have observed that the red cells are precipitated by Rivanol. The negative charge of the erythrocyte surface is due to protein carboxyl groups and sialic acid. The precipitation by Rivanol of the non-haemoglobin protein seems to indicate that the destruction of the structure of the red cell occurring during the process of haemolysis does not affect the complexes of stroma proteins with sialic acid which therefore are precipitated by Rivanol.

It seems that more numerous protein fractions obtained after removal of haemoglobin, are derived from fraction IV of the haemolysate; after Rivanol precipitation this fraction separated into ten components, and by the Hennesey & Haffner procedure [8, 9] six protein components with different mobility were obtained. Similar results were recently reported by Derrien *et al.* [4], Laurent *et al.* [14] and Markowitz [15]. Presumably fraction IV of the haemolysate is a multi-component complex the separation of which by electrophoresis is inhibited by the presence of haemoglobin. However, it cannot be excluded that in the haemolysate, haemoglobin prevents the separation of some non-haemoglobin fractions. Changes in the molecular structure of the proteins due to Rivanol treatment, should be also considered.

It seems that the use of Rivanol may prove to be an effective method for isolation and fractionation of non-haemoglobin proteins from the red blood cells.

The authors wish to express their gratitude to the Institute of Haematology, Warszawa, for supplying the preserved human blood samples.

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

SUMMARY

A method for isolation of non-haemoglobin proteins of the red blood cells is described. From the haemolysate of red cells these proteins are precipitated by Rivanol, and extracted with 0.5M-KCl; on starch-gel electrophoresis they give 13 fractions.

REFERENCES

- [1] Boettcher E., Kistler P. & Nitschmann H. - *Nature* **181**, 490, 1959.
- [2] Boyle R., Nelson S., Dollish R. & Olsen M. - *Arch. Biochem. Biophys.* **96**, 47, 1962.
- [3] Colowick S. & Kaplan N., in *Methods in Enzymology* vol. 3, p. 448. Academic Press, New York 1957.
- [4] Derrien Y., Laurent G. & Borgomano M. - *Compt. rend.* **242**, 1538, 1956.

- [5] Gorodskaja O. - *Biochimija* **15**, 507, 1950.
- [6] Haut A., Tudhope C., Cartwright G. & Wintrobe M. - *Clin. Res. Proc.* **9**, 92, 1961.
- [7] Haut A., Tudhope C., Cartwright G. & Wintrobe M. - *J. Clin. Invest.* **41**, 579, 1962.
- [8] Hennesey M. & Haffner A. - *Med. Proc.* **20**, 63, 1961.
- [9] Hennesey M., Waltersdorph A., Huennkens F. & Beverly W. - *J. Clin. Invest.* **41**, 1257, 1962.
- [10] Hořejší J. - *Čas. Lék. Česk.* **91**, 704, 1952.
- [11] Hořejší J. & Smetatana R. - *Acta Med. Scand.* **65**, 1955, 1956.
- [12] Krawczyński J. - *Ann. UMCS D. IX*, 341, 1954.
- [13] Krawczyński J. - *Pol. Tyg. Lek.* **IX**, 311, 1954.
- [14] Laurent G., Charrel M., Marric C. & Derrien Y. - *Bull. Soc. Chim. Biol.* **44**, 419, 1962.
- [15] Markowitz H., Hill A., Cartwright G. & Wintrobe M. - *Fed. Proc.* **20**, 63, 1961.
- [16] Ramsay W. - *Clin. Chem.* **2**, 221, 1957.
- [17] Saifer A. & Lipkin L. - *Proc. Soc. Exp. Biol. Med.* **102**, 220, 1959.
- [18] Saifer A., Wacsler F. & Gerstenfeld S. - *Clin. Chem.* **2**, 267, 1959.
- [19] Steidbuch M. & Quentin N. - *Nature* **183**, 323, 1959.
- [20] Tukaczyński S. & Moisiejewa W. - *Biochimija* **26**, 120, 1961.
- [21] Whitehouse M. & Moretti A. - *Biochem. J.* **86**, 3P, 1963.

IZOLOWANIE POZA-HEMOGLOBINÓWYCH BIAŁEK KRWINKI CZERWONEJ PRZY POMOCY RIVANOLU

Streszczenie

Opisano metodę izolowania pozahemoglobinowych białek krwinki czerwonej. Białka te wytrąca się z hemolizatu przy pomocy rivanolu i ekstrahuje 0.5 M-KCl. Na żelu skrobiowym uzyskano rozdział białek pozahemoglobinowych na 13 frakcji.

Received 30 December 1963.

H. von EULER

THIN-LAYER ELECTROPHORESIS OF BLOOD AND SERUM OF VARIOUS ANIMALS

Biochemical Institute, University of Stockholm, Sweden

Memorial contribution in honour of Professor Dr. Bolesław Skarżyński

It was in the year 1939, when I had the opportunity to receive Dr. Bolesław Skarżyński from Cracow in my Biochemical Research Institute, that I began our studies on the biochemistry of blood serum. Skarżyński took part in our investigations by different experiments [7, 8, 3] and studied the degradation of nucleotides and nucleic acids in liver tissue of rats [2]. In continuation of Skarżyński's experiments we found DNA implicated in mutagen actions of UV and of chemicals, and we stated the close association of DNA with genes. The structure of genes and the notion of their mutations belong to the most important problems of biochemistry.

It may be convenient to relate in this memorial volume our electrophoretic results on the components of blood of various animals, and in this way continue our investigation, initiated with Bolesław Skarżyński.

The understanding of the central role of nucleoproteins and of the fundamental biological processes must be based on the elucidation of the mode of action of their metabolic products. Therefore we early concentrated the experiments of this Institute on the study of the products of interaction between nucleosides, amino acids and phosphates. These experiments aimed at demonstrating their activity in the living organism, particularly in the chromosomes.

Which compound is responsible for the specific character of a cell? The nucleic acids and the proteins (especially the conjugated proteins) are the main or even the sole agents directing the maintenance of cellular specificity. If the structure of DNA has some direct relationship to genetic information, we must take the existence of many structural factors into consideration, in addition to Watson-Crick's analytical data on base content. The characterization and differentiation of the nucleoproteins in various animals seem desirable in accordance to the results of Hevesy & Euler [4].

Blood is one of the most essential organs of the animal body; it supplies other organs with nutrition and with oxygen, it contributes to the regulation of the acid-base balance and mediates the stability of energy. An essential and urgent problem, which we study in this connection, is concentrated to the mutagenic actions on enzymes.

By the investigation here initiated we attack a central and extremely important problem, related to anthropology, concerning the chemistry and biology of animal evolution. It points to common, not yet explained, origins of different animal groups.

Electrophoretic determination of proteins and enzymes in the blood and serum

In the present experiments, the proteins of serum or of erythrocyte haemolysate were separated by starch-gel electrophoresis according to Smithies [9] using a high-resolution buffer according to Aronson & Grönwall [1] or a borate buffer according to Thorup *et al.* [12]. The proteins were stained with Amido Black 10 B; catalase and peroxidase were located according to Thorup.

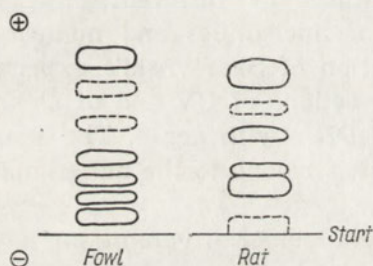


Fig. 1

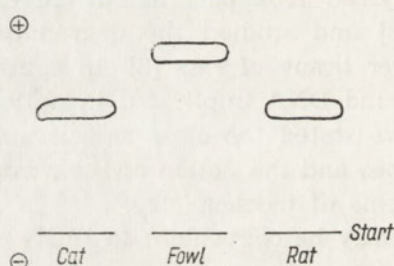


Fig. 2

Fig. 1. Electrophoresis of serum proteins of fowl and rat (white, male). Time of run: 24 hr.; voltage 6.4 v/cm.

Fig. 2. Detection of catalase, carried out by immersing the starch-gel electrophorogram in the mixture of 0.15% KI-solution + 0.85 ml. acetic acid + 0.1 ml. 3% hydroperoxide. Time of run: 20 hr.; voltage 6.5 v/cm.; 3 mA.

It was found that serum globulins from various animal species migrated with different velocities; from the diagram of electrophoretic separation of rat serum and fowl serum, presented in Fig. 1, it may be seen that fowl serum migrates faster.

Mobility of peroxidase from erythrocytes

Peroxidase was tested in the electrophoretically separated protein fractions of erythrocyte haemolysates, and its location compared with that of serum protein. 0.5 ml. of the blood was mixed with 10 ml. of

0.9% NaCl solution and centrifuged. The erythrocytes were washed with physiological saline solution, and haemolysed by adding a few drops of chloroform. The obtained clear solution was submitted to electrophoretic separation.

The peroxidases from rat, fowl and guinea pig erythrocytes migrated with different velocities, possibly reflecting the fact that the same prosthetic group is combined with different proteins. Fowl peroxidase migrated faster than that of rat or guinea pig. Human peroxidase had the velocity between that of β_1 - and γ -globulins.

Serum catalase

Serum catalases of the cat and the rat migrated at the same rate while that of the fowl migrated much faster, as may be seen in Fig. 2.

Japanese scientists Takahara [10], Kajiro, Kikuchi, Nakamura & Yoshiya [5], Nakamura, Yoshiya, Kajiro & Kikuchi [6] and Takahara, Sato, Doi & Mihara [11] reported some time ago a remarkable observa-

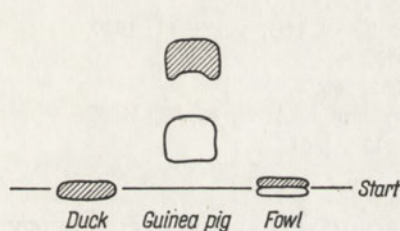


Fig. 3

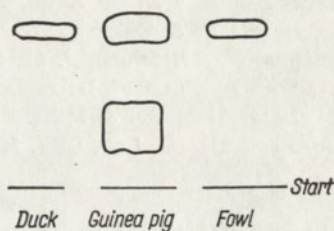


Fig. 4

Fig. 3. Catalase activity in the blood. Starch-gel electrophoresis was carried out according to Smithies [9] with borate buffer. Time of run: 4 hr.; voltage 200 v; 7 mA.

Fig. 4. Esterase activity in the blood. Conditions as for Fig. 3; elution of esterase according to Aronson.

tion, namely that blood serum of the duck contains no catalase. We wished to ascertain whether the same is true for ducks bred in Sweden; the results presented in Fig. 3 indicate that duck catalase remained on the point of application and no migration of catalase was observed.

Blood esterase

The activity of esterase was tested by spraying with β -naphthyl-propionate and detection of liberated β -naphthol with benzidin-diazonium solution according to Koch & Krieg [5a]. Fig. 4 shows that in the guinea pig two esterases are present while in the duck and fowl only one, migrating with the same velocity.

In connection with the above results it seems of great interest to continue our studies and to examine the blood of those animals which are regarded as the oldest living mammals, namely the bats (*Chiroptera*) and the hedgehog (*Erinaceus europaeus*).

SUMMARY

The rates of migration in starch-gel electrophoresis of catalase and peroxidase of some animal species were compared.

REFERENCES

- [1] Aronson & Grönwall - *Scand. J. Clin. Lab. Invest.* **9**, 338, 1957.
- [2] Euler v. H. & Skarżyński B. - *Z. physiol. Chem.* **263**, 259, 1940.
- [3] Euler v. H. & Skarżyński B. - *Arkiv Kemi* **17B**, no. 15, 24, 1943/1944.
- [4] Hevesy G. & Euler v. H. - *Arkiv Kemi* **17A**, no. 31, 1942.
- [5] Kajiro K., Kikuchi G., Nakamura H. & Yoshiya K. - *Chem. Ber.* **85**, 886, 1952.
- [5a] Koch J. E. & Krieg W. - *Chemiker Z.* **62**, 140, 1938.
- [6] Nakamura, Yoshiya, Kajiro & Kikuchi - *Proc. Japan. Acad.* **28**, 59, 1952.
- [7] Skarżyński B. - *Arkiv Kemi*, **13B**, no. 13, 1940.
- [8] Skarżyński B. & Euler v. H. - *Arkiv Kemi* **14B**, no. 3, 11, 1940.
- [9] Smithies O. - *Biochem. J.* **61**, 629, 1955.
- [10] Takahara S. - *Lancet*, December 6, 1101, 1952.
- [11] Takahara, Sato, Doi & Mihara - *Proc. Japan. Acad.* **28**, 585, 1952.
- [12] Thorup et al. - *J. Lab. Clin. Med.* **58**, 122, 1961.

ELEKTROFOREZA NA ŻELU SKROBIOWYM KRWI I SUROWICY RÓŻNYCH ZWIERZĄT

Streszczenie

Porównano szybkość wędrowania katalazy i peroksydazy różnych zwierząt w elektroforezie na żelu skrobiowym.

Received 3 January 1964.

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STUDIES ON THE ADMINISTRATION OF RIBOFLAVIN-5'-MONOSULPHATE

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Dedicated to the late Professor Dr. Bolesław Skarżyński

As reported previously, FMS¹ has been synthesized as a coenzyme analogue [4] and has been found to inhibit D-amino acid oxidase in competition with FAD [2, 9, 10]. On the other hand, it has been observed that FMS has an inhibitory action on the growth of riboflavin requiring bacteria [1].

From these results, it was supposed that FMS, as an antivitamin B₂, disturbs the metabolic pattern in higher animals. To study this anti-vitamin action of FMS, the effect of its administration to animals may be observed by physiological and nutritional experiments. In this connection, we tried to establish a method for the determination of FMS in animal tissues. This paper reports a method for the micro-determination of flavins including FMS and distribution of flavins in rat organs after administration of FMS as well as the results of nutritional experiments on FMS.

MATERIALS AND METHODS

Preparation of FMS. FMS was synthesized according to the method of Takahashi *et al.* [4].

Distribution of flavins in organs after administration of FMS. To investigate the distribution of flavins after administration of FMS, male albino rats weighing about 130 g. were used. The animals were injected intraabdominally with 500 µg. of FMS and killed at a definite time after the injection. The organs were excised immediately and the quantity of each flavin compound was determined.

¹ Following abbreviations are used: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FR, riboflavin; FMS, riboflavin-5'-monosulphate.

Determination of FMS in animal tissues. The previously reported method [5] for separate determination of flavins could be adopted for the determination of FMS, FAD, FMN and FR by changing the solvent system for paper chromatography. Among solvent systems tested, *n*-butanol - acetone - acetic acid - water (5:2:1:3, by vol.) was found to be most suitable to separate these flavins from each other [11]. Using this solvent system and Whatman no. 1 filter paper, the R_F values of FAD, FMS, FMN and FR were 0.08, 0.15, 0.25 and 0.42, respectively. Thus, the following standard procedure was established.

Tissue is excised from animals, immediately weighed (x g.), cut into small pieces and put into 5 ml. of water previously warmed at 80° and then kept at 80° for 5 min. Then, the tissue is homogenized in a glass homogenizer, diluted with water and warmed again at 80° for 15 min. The total volume of the extract is made up to y ml. exactly with water and an aliquot of the supernatant is used as test solution for lumiflavin fluorescence method to obtain the total flavin content ($\mu\text{g./g.}$) of the tissue. The remaining part of the warm water extract of the tissue is saturated with ammonium sulphate and centrifuged. The supernatant fluid is extracted twice each time with 2 ml. of phenol. The flavins in the combined phenol layer are transferred to few drops of water by addition of 20-30 ml. of ethyl ether and the water extract of flavins is placed on a filter paper. After development with *n*-butanol - acetone - acetic acid - water (5:2:1:3, by vol.), each spot area is clipped, cut into small pieces, suspended in 2.0 ml. of water, added with 2.0 ml. of 1 N-NaOH [7], followed by the irradiation and successive procedures of lumiflavin fluorescence method.

The amount of each flavin is calculated by the following formulas, a , b , c and d being the reading of fluorimeter for FMS-, FAD-, FMN-, and FR-sample.

$$\text{FMS} = f \times \frac{a}{a+b+c+d} (\mu\text{g./g.})$$

$$\text{FAD} = f \times \frac{b}{a+b+c+d} (\mu\text{g./g.})$$

$$\text{FMN} = f \times \frac{c}{a+b+c+d} (\mu\text{g./g.})$$

$$\text{FR} = f \times \frac{d}{a+b+c+d} (\mu\text{g./g.})$$

These values are expressed in terms of FR. When absolute amounts are required, these values should be multiplied by 1.21, 2.09, 1.21 and 1.0, respectively.

Excretion of FMS in urine. To examine the excretion of FMS in urine, 500 μ g. of FMS were given by intraabdominal injection or by oral administration and flavins excreted in urine were determined using the paper chromatography as mentioned above.

Changes in body weights of rats after administration of FMS. For nutritional experiments, male rats weighing about 100 g. were fed previously on a basal diet [3, 8] for a week. Twenty rats were divided into 4 groups of 5 rats. Group 1 was fed on the basal diet as the control and group 2 received vitamin B₂-deficient diet. Group 3 received vitamin B₂-deficient diet and was injected intraabdominally with 500 μ g. of FMS and 10 μ g. of FR per day. Group 4 received vitamin B₂-deficient diet, and 500 μ g. of FMS and 10 μ g. of FR per day were given compulsorily by oral administration. The changes in weights of animals were observed for 30 days.

RESULTS

Distribution of flavins after administration of FMS. The amounts of flavins in the liver, kidney, heart and small intestine were determined with normal rats and those at 30 min. 1, 2, 6 and 12 hr. after the injection of FMS. The results are summarized in Table 1. The maximum amount of FMS in the liver, kidney or heart was found at 30 min. after the injection. Then the amount decreased gradually with time, and almost all of FMS in these organs disappeared after 12 hr. During this time, no remarkable change was observed in the amount of FAD, FMN and FR, and the increase of the total flavin content could be attributed to the appearance of FMS. In the small intestine the amount of FMS reached its maximum at 60 min. after the injection.

Excretion of administered FMS in urine. After FMS was injected intraabdominally, the excreted flavins in urine were analysed by the above-mentioned paper chromatography. The most part of the flavins in urine was FMS, and the patterns of excretion of FMS in urine were summarized in Table 2. About half of the injected FMS was excreted in 1 hr. and over 90% was recovered in 12 hr. On the other hand, FMS given by oral administration appeared in urine much more slowly.

Effects of the administration of FMS on body weight of rats. The body weight of rats of four groups was measured for 1 month. The body weight of group 1 which was fed on the standard diet increased smoothly till the end of the test as shown in Fig. 1. In group 2, which was fed on vitamin B₂-deficient diet, the body weight increased in the first week and then gradually decreased as shown in Fig. 2. One of the rats in group 2 died after 28 days. Group 3, which received FMS together with FR by injection, showed the similar tendency in body weight change

Table 1

Distribution of flavin compounds in organs of rats after the injection of FMS

Each value, which is expressed in terms of FR, is the mean obtained from 5 experiments.

Tissue	Time after the injection (hr.)	Total flavin ($\mu\text{g./g.}$)	FAD ($\mu\text{g./g.}$) (%)	FMN ($\mu\text{g./g.}$) (%)	FMS ($\mu\text{g./g.}$) (%)	FR ($\mu\text{g./g.}$) (%)
Liver	0	29.5	22.1 75.0	6.7 22.7	0 0	0.7 2.3
	0.5	33.2	23.2 69.9	6.1 18.4	3.1 9.3	0.8 2.4
	1	29.0	20.5 70.7	6.7 23.1	1.2 4.1	0.6 2.1
	2	26.9	19.5 72.5	5.7 21.2	0.9 3.3	0.8 3.0
	6	30.3	22.7 75.0	6.2 20.4	0.6 2.0	0.8 2.6
	12	30.7	22.0 71.7	7.5 24.4	0.5 1.6	0.7 2.3
Kidney	0	34.9	24.1 69.0	9.9 28.4	0 0	0.9 2.6
	0.5	41.8	23.6 56.5	9.3 22.2	7.9 18.9	1.0 2.4
	1	38.0	22.8 60.0	10.7 28.1	3.4 9.0	1.1 2.9
	2	34.9	24.7 70.8	8.7 24.9	0.6 1.7	0.9 2.6
	6	33.1	21.2 64.0	10.6 32.1	0.5 1.5	0.8 2.4
	12	33.9	23.1 68.1	9.7 28.6	0.2 0.6	0.9 2.7
Heart	0	15.9	14.3 90.0	1.5 9.7	0 0	0.1 0.3
	0.5	17.9	13.5 75.5	1.7 9.5	2.4 13.4	0.3 1.6
	1	19.1	15.9 83.4	2.2 11.4	0.8 4.2	0.2 1.0
	2	15.5	13.5 87.1	1.5 9.7	0.5 3.2	0* 0
	6	17.4	15.0 86.3	1.7 9.8	0.6 3.4	0.1 0.5
	12	14.9	13.3 89.3	1.6 10.7	0 0	0* 0
Small intestine	0	4.3	3.2 74.5	1.0 23.0	0 0	0.1 2.5
	0.5	8.4	3.9 46.4	1.6 19.0	2.4 28.6	0.5 6.0
	1	9.6	2.5 26.0	1.8 18.7	5.3 55.3	0* 0
	2	4.7	3.2 68.1	1.0 21.3	0.3 6.4	0.2 4.2
	6	4.5	3.0 66.7	0.8 17.8	0.4 8.9	0.3 6.6
	12	4.4	2.8 63.6	1.1 25.0	0.3 6.8	0.2 4.6

* Unmeasurably small.

Table 2

Excretion of administered FMS in urine

Injection				Oral administration			
Time after the injection (hr.)	Urine volume (ml.)	FMS ($\mu\text{g./ml.}$)	Total amount of FMS ($\mu\text{g.}$)	Time after the oral administration (hr.)	Urine volume (ml.)	FMS ($\mu\text{g./ml.}$)	Total amount of FMS ($\mu\text{g.}$)
0 - 0.5	0.1	1260	126	0 - 6	0.2	347	69
0.5 - 1	0.1	940	94	6 - 12	0.3	297	90
1 - 6	0.25	648	162	12 - 25	0.5	442	221
6 - 12	0.4	225	90				
Total	0.85		472	Total	1.0		380

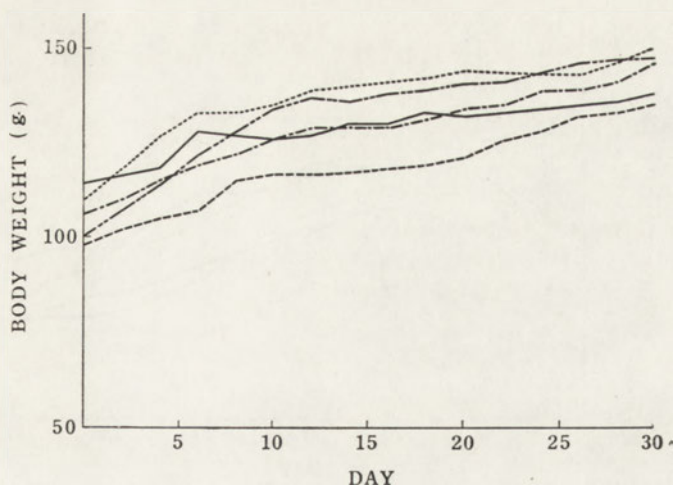


Fig. 1. Changes in body weights of rats receiving the standard diet.

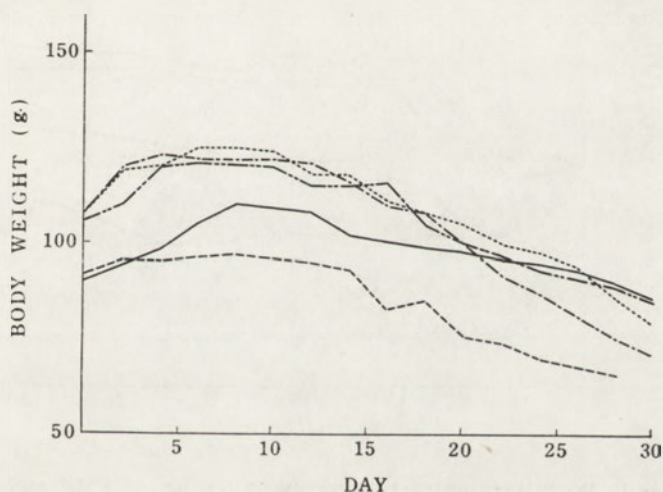


Fig. 2. Changes in body weights of rats receiving the vitamin B₂-deficient diet.

to group 2 (see Fig. 3). On the contrary, no decrease but increase of body weight was observed in group 4 receiving FMS and FR by oral administration (see Fig. 4). Only the rats in group 2 and 3 showed the typical vitamin B₂-deficiency symptoms such as nose-bleeding, moistening of hair, swelling of ears, inflammation in eyelids and falling off of hair.

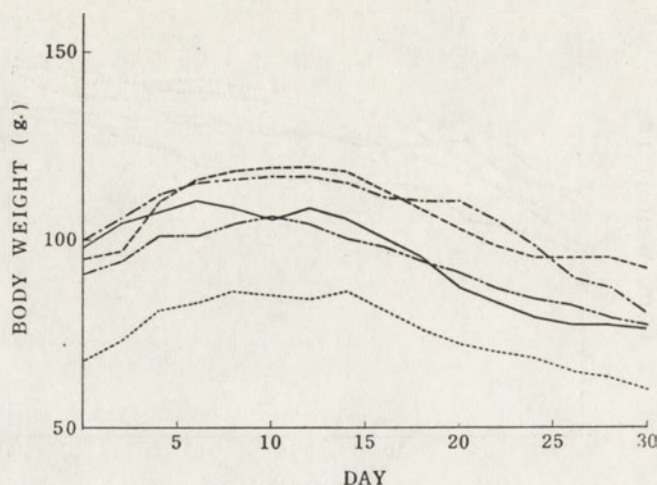


Fig. 3. Changes in body weights of rats receiving 500 μ g. of FMS and 10 μ g. of FR per day by injection.

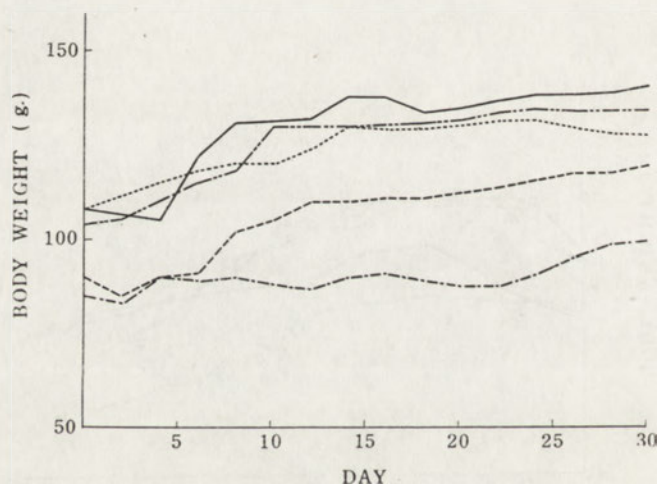


Fig. 4. Changes in body weights of rats receiving 500 μ g. of FMS and 10 μ g. of FR per day by oral administration.

DISCUSSION

The contents of flavins in the kidney, heart and small intestine of normal rats are very similar to those reported in the previous paper [6]. When rats were injected with FMS, the FMS was demonstrated in these organs at 30 min. after the injection and almost disappeared after 12 hr. During this time, the amounts of FAD, FMN and FR in these organs were not changed. Consequently, it is considered that physiologically

existing flavins in these organs might not be exchanged for the injected FMS.

In the small intestine at 1 hr. after the injection of FMS, relatively large amount of FMS as compared with the amount of other physiologically existing flavins was observed. Considering the fact that FR injected gathers in the mucous membrane of the small intestine [6], the same mechanism may be considered as for the injected FMS. The results of the analysis indicate that only 6% of the injected FMS was found in the above-mentioned organs and that most part of the injected FMS was excreted in urine very rapidly in the form of FMS.

From the results of nutritional experiments, it is obvious that FMS acts as an antivitamin B₂. It seems that FMS competes with flavin-coenzyme in living cells [2, 9, 10] but it is excreted in urine so rapidly that it shows no serious toxicity. In the case of oral administration of FMS, we have observed the body weight gain and no ariboflavinosis. To explain this fact, two possibilities may be speculated, i.e., (1) relatively slower absorption of FMS from intestine than FR, (2) conversion of FMS to FR by some intestinal bacteria. These possibilities should be examined further.

It may be noted that FMS, the competitive inhibitor of flavin enzymes, acts as an antivitamin not only on bacteria but also on rats.

SUMMARY

1. A method for the determination of flavins including FMS in animal tissues was reported.

2. The distribution of flavins after administration of FMS was determined. Injected FMS was demonstrated in the liver, kidney, heart and small intestine at 30 min. after the administration and almost disappeared after 12 hr. After the injection of FMS, the amounts of FAD, FMN and FR in these organs were not changed.

3. Excretion of administered FMS in urine was observed. Over 90% of the injected FMS was recovered in 12 hr. and in the case of oral administration, about 75% of the administered FMS was recovered in 25 hr.

4. In the nutritional experiments, injected FMS showed an antivitamin B₂ effect, but FMS administered orally did not show such an effect.

REFERENCES

- [1] Egami F., Naoi M., Tada M. & Yagi K. - *J. Biochem. (Tokyo)* **43**, 310, 1960.
- [2] Egami F. & Yagi K. - *J. Biochem. (Tokyo)* **43**, 153, 1956.
- [3] Forker B. R. & Morgan A. F. - *J. Biol. Chem.* **209**, 303, 1954.
- [4] Takahashi N., Yagi K. & Egami F. - *J. Chem. Soc. Japan, Pure Chem. Sect.* **78**, 1287, 1957.

- [5] Yagi K. - *J. Biochem. (Tokyo)* **38**, 161, 1951.
- [6] Yagi K. - *J. Biochem. (Tokyo)* **41**, 757, 1954.
- [7] Yagi K., Kondo H. & Okuda J. - *J. Biochem. (Tokyo)* **51**, 231, 1962.
- [8] Yagi K., Okuda J. & Kobayashi M. - *J. Vitaminol.* **9**, 168, 1963.
- [9] Yagi K. & Ozawa T. - *Biochim. Biophys. Acta* **39**, 304, 1960.
- [10] Yagi K. & Ozawa T. - *Biochim. Biophys. Acta* **42**, 381, 1960.
- [11] Yagi K. & Yamada S. - *Nagoya J. Med. Sci.* **25**, 228, 1963.

BADANIA NAD 5'-MONOSIARCZANEM RYBOFLAWINY

Streszczenie

1. Opisano metodę oznaczania FAD, FMN, ryboflawiny i 5'-monosiarczanu ryboflawiny (FMS) w tkankach zwierzęcych.

2. Oznaczono rozmieszczenie flawin w wątrobie, nerce, sercu i jelicie cienkim szczura. Po iniekcji FMS ilości FAD, FMN i ryboflawiny nie uległy zmianie. W 30 min. po zastrzyku FMS był obecny w badanych tkankach; po 12 godz. nie stwierdzono jego obecności.

3. Badano wydalanie w moczu podanego FMS. Po iniekcji ponad 90% FMS odnajdywano w ciągu 12 godz., po doustnym podaniu — ok. 75% w ciągu 25 godz.

4. FMS podawany pozajelitowo wykazywał własności antywitaminy B₂, podawany doustnie nie miał takich własności.

Received 3 January 1964.

A. BRZEZIŃSKI and B. FILIPOWICZ

**THIAMINE AND THIAMINE ESTERS IN THE GUINEA PIG LIVER
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Many experimental works as well as clinical observations suggest disturbances in carbohydrate metabolism in diphtheria. These are manifested also by the increase of pyruvic acid content in the blood [9, 1]. The mortality in diphtheria due to cardiac lesions decreased since treatment with cocarboxylase has been introduced [9]. In experimental diphtherial toxæmia in the guinea pig, beside an increase of pyruvic acid in the blood, we have found a marked decrease of cocarboxylase content in the liver [18]. The decrease of cocarboxylase could be due to its impaired synthesis or to increased dephosphorylation. It was the aim of the present work to elucidate this problem.

MATERIAL AND METHODS

Male guinea pigs, kept on a normal mixed diet given *ad libitum* and weighing about 250 g., received subcutaneously approx. 1 DLM of diphtherial toxin. 48-60 hr. after the intoxication the animals, showing already the symptoms of illness (apathy, tousled hair, lack of appetite, loss of weight) were used for experiments.

Thiamine was estimated by the Hennessy-Cerecedo method with the modifications of Hennessy [3] and Mickelsen & Yamamoto [11]. In this method, thiamine is oxidized to thiochrome which is then extracted with isobutanol and estimated fluorometrically. As only free thiochrome is extracted by isobutanol, for the estimation of thiamine phosphates (TMP¹, TDP and TTP) the sample before oxidation is treated with phosphatases. From the values of free and total thiamine, the amount of thiamine phosphates is calculated.

The animals were decapitated, the liver immediately removed, immersed in cold 1.22% KCl solution, and washed. Then 10% water homogenate was prepared in a Waring blender. Homogenization time was

¹ TMP, TDP and TTP, thiamine mono-, di- and triphosphate, respectively.

1.5 min., with a break after 45 sec. for cooling the material. All procedures were performed in a cold room at 0–4°. An amount of the homogenate corresponding to 1 g. of fresh tissue was used for the estimation of total thiamine, and to 2 or 3 g., for free thiamine estimation. All samples were estimated in duplicate.

Aliquots of the homogenate were diluted with water, acidified with 2 N-hydrochloric acid and placed for 30 min. in a boiling water bath. After cooling, the samples in which total thiamine was to be estimated were hydrolysed with 6% solution of Taka-Diastase (K & K Lab., U.S.A.) in 2.5 M-solution of CH_3COONa . To the samples in which free thiamine was to be estimated only CH_3COONa was added. After 3 hr. incubation at 50° the samples were filtrated through Whatman no. 1 paper, adjusted, if necessary, to pH 4.0–4.5 and placed on columns containing activated Decalso [14]. The columns were washed with hot water and then thiamine was eluted with boiling 25% (w/v) solution of KCl in 0.1 N-hydrochloric acid. Under these conditions, 92–103% of the introduced vitamin was recovered. From the KCl eluates blank samples and samples in which thiamine was oxidized with alkaline ferricyanide [11] were prepared. The formed thiochrome was extracted with isobutanol and determined on Pulfrich photometer with fluorometric set (Filter L3). The concentration of thiamine in the sample was calculated from the standard curve prepared every day in the same way as the tested samples.

RESULTS AND DISCUSSION

No statistically significant differences were found in the amounts of free and total thiamine between the control and intoxicated animals (Table 1). It may be, however, that the time of intoxication was too short for the development of thiamine deficiency. In the previous work [18], under the same experimental conditions, a marked decrease of co-carboxylase in the liver of intoxicated guinea pigs was found by the enzymic method of Westenbrink & Steyn-Parvé [19]. As at the same time no change was found in the content of thiamine phosphates, the decrease of cocarboxylase can be accounted for by formation of TMP and/or TTP.

The content of cocarboxylase found in normal liver agreed with the amount of esterified thiamine, suggesting that all or almost all esterified thiamine is present in the guinea pig as thiamine diphosphate. This is in agreement with the data of Foa *et al.* [2], Rindi & de Giuseppe [12] and Kiessling [6] indicating that in the rat body almost all thiamine is present in this form.

In the liver of intoxicated animals the amount of cocarboxylase was smaller by about 20% than that of esterified thiamine. The difference between these two values, corresponding to 2.17 μm -moles/g. fresh tissue,

Table 1

Content of free and esterified thiamine and of cocarboxylase in the liver of normal and diphtheria-intoxicated guinea pig

Total and free thiamine was determined fluorometrically [11], and cocarboxylase enzymically [19]. The figures represent $\mu\text{m-moles per g. fresh tissue}$. Mean values, $\pm \text{S.D.}$ In parentheses the number of determinations. *P* values according to Student's *t* test.

Compound	Control animals	Intoxicated animals	<i>P</i>
Total thiamine	12.98 \pm 2.73 (13)	11.68 \pm 2.81 (11)	> 0.3
Free thiamine	1.42 \pm 0.68 (13)	0.97 \pm 0.49 (11)	> 0.3
Esterified thiamine	11.55 \pm 2.40 (13)	10.71 \pm 2.48 (11)	> 0.3
Cocarboxylase*	11.71 \pm 0.53 (10)	8.54 \pm 0.41 (8)	< 0.001

* Results according to [18].

was probably due to the enzymically inactive TMP or TTP. The formation and the role of TTP is still unknown and the accumulation of TMP seems to be more probable. An enzyme dephosphorylating TDP to TMP was described by Tilander & Kiessling [15] and increased activity of this enzyme, followed by an increase of TMP at the expense of TDP were found in the liver of rats intoxicated with CCl_4 [7] or ethanol [8] and in alloxan diabetes [5]. It seems that the liver of a guinea pig responds to diphtherial toxin by an analogous increase of phosphatase activity. This assumption is supported also by the experiments of Witkowski & Filipowicz [17] who under similar conditions of diphtherial toxæmia found in guinea pig liver homogenates increased dephosphorylation of the added TDP. However, they did not identify the decomposition products.

The cocarboxylase decrease caused by the diphtherial toxin could be also explained by impaired phosphorylation of thiamine. However, this should be accompanied by an increase of free thiamine, and this was not the case. In normal and intoxicated guinea pig liver the content of free thiamine amounted to 10% of the total. Recently Witkowski [16] also reported that in diphtherial toxæmia no changes in thiaminepyrophosphokinase activity were observed.

Although it is well known [10, 13, 4] that TDP is formed from thiamine by pyrophosphotransferase reaction, the occurrence of another synthesis: $\text{T} + \text{ATP} \rightarrow \text{TMP} + \text{ADP}$; $\text{TMP} + \text{ATP} \rightarrow \text{TDP} + \text{ADP}$ may be also considered. This way of TDP formation might be stimulated in some circumstances.

SUMMARY

In the liver of a normal guinea pig the content of esterified thiamine (about 90% of total thiamine) corresponds to the level of cocarboxylase. In experimental diphtherial toxæmia no changes in the content of total and esterified thiamine were found; the observed decrease of cocarboxylase content by 27% may be explained by the increased activity of thiamine pyrophosphatase causing formation of thiamine monophosphate.

REFERENCES

- [1] Filipowicz B., Redlich F., Margolis A. & Witkowska Z., *Pamiętnik Zjazdu Pediatrów*, p. 82, Szczecin 1956.
- [2] Foa P. P., Weinstein H. R., Smith J. A. & Greenberg M. - *Arch. Biochem. Biophys.* **40**, 323, 1952.
- [3] Hennessy D. J. - *Ind. Eng. Chem., Anal. Ed.* **13**, 215, 1941, cited after [14].
- [4] Kazi Y., Ryo T., Yoshitaka M. & Norio S. - *J. Biochem. (Tokyo)* **49**, 472, 1961, cited after Ref. *Żurnal* **2C267**, 1962.
- [5] Kiessling K. H. - *Biochim. Biophys. Acta* **39**, 412, 1960.
- [6] Kiessling K. H. - *Biochim. Biophys. Acta* **46**, 603, 1961.
- [7] Kiessling K. H. & Tilander K. - *Biochim. Biophys. Acta* **43**, 335, 1960.
- [8] Kiessling K. H. & Tilander K. - *Exptl. Cell Res.* **19**, 3, 1960.
- [9] Lasch F. - *Dt. Med. Wschr.* **27/28**, 975, 1953.
- [10] Leuthardt F. & Nielsen H. - *Helv. Chim. Acta* **35**, 1196, 1952.
- [11] Mickelsen O. & Yamamoto R. S., in *Methods of Biochemical Analysis* (D. Glick, ed.) **6**, 191, 1958.
- [12] Rindi G. & de Giuseppe L. - *Biochem. J.* **78**, 602, 1961.
- [13] Rossi-Fanelli A., Siliprandi N., Fasella P., Siliprandi D. & Salvetti M. - *Experientia* **10**, 73, 1954.
- [14] Stiller E. T., in *Vitamin Methods*, vol. I, p. 98, Academic Press, New York 1950.
- [15] Tilander K. & Kiessling K. H. - *Acta Chem. Scand.* **15**, 477, 1961.
- [16] Witkowski S. - *Arch. Immunol. Therap. Exptl.* **12**, 1, 1964.
- [17] Witkowski S. & Filipowicz B. - *Experientia* **20**, 72, 1964.
- [18] Witkowski S., Brzeziński A., Galamon T. & Filipowicz B., *Intern. Symposium on B Vitamins*, p. 273, Poznań 1959.
- [19] Westenbrink H. G. K. & Steyn-Parvé E. P. - *Int. Z. Vitaminforsch.* **21**, 461, 1950.

TIAMINA I JEJ ESTRY W WĄTROBIE ŚWINKI MORSKIEJ
W DOŚWIADCZALNEJ TOKSEMII BŁONICZEJ

Streszczenie

W wątrobie zdrowych świnek morskich zawartość tiaminy ufosforylowanej (około 90% tiaminy całkowitej) odpowiada poziomowi kokarboksylazy. W eksperymentalnej toksemii błoniczej nie zauważono zmian w zawartości tiaminy całkowitej i zestryfikowanej. Obniżony o około 27% poziom kokarboksylazy można tłumaczyć wzrostem aktywności pirofosfatazy, rozkładającej ester dwufosforowy do estru monofosforowego.

Received 3 January 1964.

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γ -AMINOBUTYRIC ACID CONTENT IN RAT BRAIN PROCESSED UNDER DIFFERENT TEMPERATURE CONDITIONS

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The discovery of γ -aminobutyric acid (GABA) in the brain [3, 15, 23] posed new problems for biochemists and neurophysiologists. This compound, as an α -decarboxylation product of an amino acid, is included among the biogenic amines similarly as catecholamines and serotonin. The inhibitory effect of exogenous GABA on neuronal activity has suggested its participation in synaptic transmission, either as an activator, or transmitter, of inhibitory impulses, or as a blocking agent for excitatory synapses, in invertebrates [6, 9] and in mammals [8, 11, 17, 13, 24]. As GABA attains in the brain much higher levels than other biogenic amines, it may be more generally involved in the metabolism and energy supply [18, 13] of the central nervous system.

It is well known that the amounts of free or easily extractable amino acids change in tissues with time after death. A relative increase is especially pronounced in the case of those amino acids which represent only a minor constituent of the amino acid fraction. This is accounted for by continuation of the *in vivo* proteolysis, no more balanced by protein synthesis, as well as liberation of proteolytic enzymes from lysosomes [2]. In our experiments, when the brain had been frozen immediately after the death of the animal, leucines and valine were hardly detectable by the methods employed, but they could be readily detected and quantitatively estimated if the brain had been kept for 3 hr. at room temperature. Also the amount of GABA increased considerably, although this compound has not been detected in brain protein.

Roberts *et al.* [16, 17] found that the amount of GABA in the brain changes during the development of the animal. We are planning to study changes in the metabolism of endogenous amines, especially of GABA, during ageing; for this purpose, it is essential to obtain values most closely corresponding to those which prevail *in vivo*, and to minimize the *post-mortem* increase in GABA content. Therefore, in the present

work four different methods of treatment of the head of the killed rat were studied. It was found that the treatment with liquid nitrogen gave the lowest GABA values.

The freezing of tissues with liquid oxygen or nitrogen is frequently used to stop the metabolic processes. The question arose to what extent this treatment limited the increase of GABA against the *in vivo* levels. Therefore in the present experiments the changes in temperature of the brain during freezing were also studied. Information about the zero-time values was sought by following the course of temperature drop within the brain, using two freezing media. In small animals or organs the metabolic process can be expected to stop within a few seconds, whereas in relatively bulky objects with high specific heat, enzymic activity may proceed for some time after the organ has been immersed in the freezing medium.

MATERIALS AND METHODS

Animals. Wistar albino rats of the local substrain, of both sexes, weighing 150 - 200 g., which had been kept on a modified Larsen diet, were used. In the morning hours, the animals were decapitated with the use of scissors, and the heads submitted to one of the following four treatments: (A), immediate immersion into liquid nitrogen; (B), immediate immersion into a mixture of solid carbon dioxide and ethanol; (C), removal of the brain and immediate deproteinization with ethanol at room temp.; (D), standing at room temp. for 30 min. before deproteinization.

Freezing and isolation of the brain. The CO₂-ethanol cooling mixture was prepared by mixing pieces of dry ice with ethanol in a Dewar flask. After 5 min. of freezing, the head was chopped into two halves, the cranium removed with a chisel and the brain taken out. The brain was hard and firm, with slightly moist surface. If the head was immersed in liquid nitrogen, after about 1 min. the cranial base cracked loudly into two halves. After 3 min. the head was taken out, and the brain was removed. The brain was hard and fragile.

Homogenization and extraction. The brain (about 1 g.) was put into a weighed test tube containing 10 ml. of 75% ethanol cooled to about -10°, weighed and homogenized for 3 min. in a motor-driven Potter-Elvehjem homogenizer fitted with a Umaplex pestle. The suspension was centrifuged and 5 ml. of the supernatant was evaporated on a porcelain dish under an infra-red lamp in a stream of air [18]. The residue was dissolved in 1 ml. of aqueous propan-2-ol (10%), centrifuged, and two 0.05 ml. aliquots of the supernatant corresponding to about 25 mg. of brain tissue were submitted to paper chromatography.

Paper chromatography and estimation of GABA. GABA standard was dissolved in 10% aqueous propan-2-ol to make a 0.02% solution, and

5 or 10 $\mu\text{g.}$, depending on the expected amount of GABA, were chromatographed together with the sample. The butan-1-ol - acetic acid - water (4:1:5, by vol.) solvent system within 2 days after preparation, and Whatman no. 1 paper were used. For the estimation of GABA, the chromatogram was developed once and for other amino acids, three times. After being dried in a current of warm air (5 min. at 45°) the chromatograms were immersed in 0.5% ninhydrin solution in acetone (anal. grade). This reagent was stored in a refrigerator at 2° for not more than a week, but no deterioration was observed even after 3 weeks. After evaporation of acetone, the chromatogram was heated at $60^\circ \pm 1^\circ$ for exactly 30 min. The GABA spots and the corresponding areas of non-stained paper were cut out, extracted with 4 ml. of methanol containing a cupric salt (1 ml. of a saturated aqueous solution of copper nitrate in 1 litre methanol), and left in the dark with occasional shaking. After 1 hr. the extinction of the extract was measured at 500 $\text{m}\mu$ in a test tube of 1 cm. diameter, using the photometer K-4 (Prema). The colour was stable for at least 3 hr.

Temperature changes during freezing. A thermocouple was made by fusing together the ends of a constantan and copper wires (diameter 0.5 mm., lacquer-insulated). The thermocouple was inserted into the brain through the *foramen occipitale* and situated at the level of the *diencephalon*. The skin of the neck was tightly bound around the thermocouple wires. A wooden rod was fixed to the head, parallel with its axis, by means of an adhesive tape (Isolepa) and the thermocouple wires were attached to the rod with the same tape to immobilize the thermocouple. To prevent the access of the cooling liquid through the hole pierced with the thermocouple, an ink mark was made on the rod to indicate how far the head should be lowered into the liquid. Two wires attached to the ears served to suspend the head on the rim of the Dewar flask. The head was then immersed into the liquid exactly to the level of the ears and galvanometer readings were taken in 15 sec. intervals. The deflexions of the Interflex galvanometer were previously standardized to temperature with an alcohol thermometer.

Since in this experiment the head was not wholly immersed, the cranial base did not split into two halves in liquid nitrogen; there was, however, a crack after 45 - 60 sec., followed by a series of lighter cracks during the following minutes.

RESULTS

The accuracy of the analytical method. The smallest amount of GABA which could be determined was about 1 $\mu\text{g.}$ per spot. The plot of extinction versus amount of GABA was linear up to 10 $\mu\text{g.}$ The slope differed within about 10% in repeated assays. When known amounts of the

standard were added to the samples of the analysed brain extracts, recoveries were unaffected by the constituents of the extracts. This has justified the calculation of GABA content on the basis of proportionality, from the extinction values for standards run on the same paper sheet, after correcting for paper background. The extinctions of identical standard samples run on the same paper sheet varied by less than 2%. When 10 aliquots of the same brain extract were run on different sheets, the coefficient of variation (standard deviation expressed as a percentage of the mean) was 1.2%.

GABA content in brain after different treatments. The mean values and standard deviations are summarized in Table 1 and illustrated in

Table 1

Determination of GABA in rat brain

The results are expressed as $\mu\text{g. per g. of fresh tissue}$, $\pm \text{S.D.}$; in parentheses the limit values are given.

Treatment	No. of animals	GABA content	Authors
A, Head decapitated, frozen in liquid nitrogen	25	185 ± 16 (156 - 214)	Present experiments
B, Head decapitated, frozen in $\text{CO}_2\text{-EtOH}$	16	212 ± 17 (193 - 241)	Present experiments
C, Head decapitated, brain immediately deproteinized at room temp.	15	304 ± 23 (266 - 348)	Present experiments
D, Head decapitated, brain deproteinized after 30 min. at 20°	7	276 ± 28 (441 - 506)	Present experiments
Whole animal frozen in liquid oxygen	10	157 ± 23	Sotnikova & Sytinskiĭ [20]
Whole animal frozen in liquid oxygen	12	189 ± 13 (162 - 204)	Musaelyan & Sytinskiĭ [10]
Head decapitated, frozen in liquid oxygen	5	206 (162 - 279)	Ansell & Richter [1]
Brain immediately deproteinized at room temperature		$282 \pm 24^*$	Turský & Šajter [21, 22]
Brain deproteinized within a few min. after killing of the animal		633**	Awapara <i>et al.</i> [3]

* Standard error.

** Approximate value.

Fig. 1. The difference between the mean values found after freezing with liquid nitrogen and with CO_2 -ethanol was subjected to Student's t test. The probability that the difference is due to chance was less than 0.1% ($t = 5.05$ for 39 D.F.).

Time course of the temperature decrease in the brain during freezing. Fig. 2 shows the temperature course after immersion of the head in

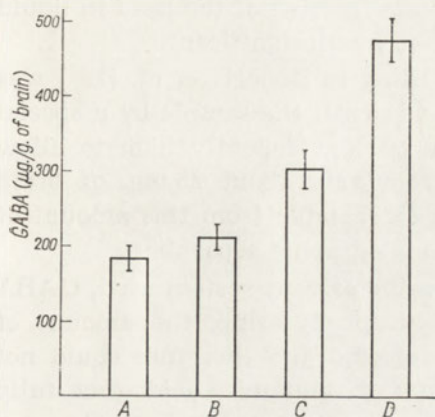


Fig. 1. γ -Aminobutyric acid content in rat brain as found after different treatments after decapitation. (A), Head immediately immersed in liquid nitrogen for 3 min.; (B), head immediately immersed in CO_2 -ethanol for 5 min.; (C), brain deproteinized immediately without freezing; (D), brain deproteinized after 30 min. at room temp. Average values, \pm S.D., are given.

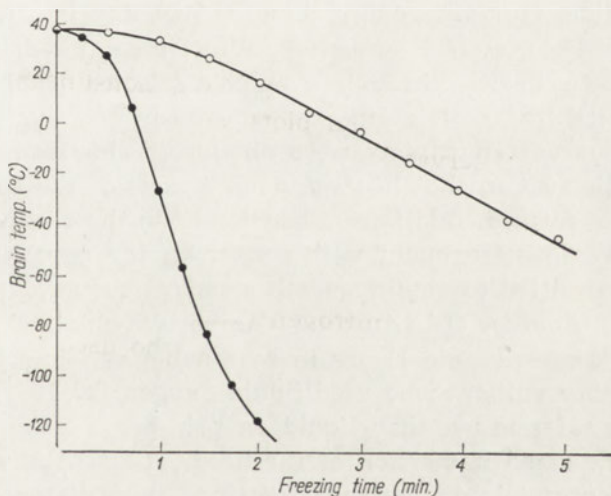


Fig. 2. Temperature in the hypothalamic region after immersion of the head in the freezing liquid. Averages from 3 determinations. (○), CO_2 -ethanol mixture, -75° ; (●), liquid nitrogen.

liquid nitrogen or in CO_2 -ethanol mixture. In the case of liquid nitrogen it is probable that the steep part of the curve below 0° corresponds to the stage when the cooling liquid could penetrate through the cracks. In the experiments for GABA determination, when the whole head was submerged in the liquid and the cranial base became widely split, the cooling effect after about a minute might have been even more marked.

DISCUSSION

Decapitation. The method of killing may affect the amount of some metabolites in the brain [12]. This has not been investigated in the present study, but Ansell & Richter [1] found, for the amino nitrogen content of brain, that the difference between decapitation (a blow with a hammer on a razor blade) with immediate freezing of the head in liquid oxygen, and freezing of the whole animal was insignificant.

Homogenization. Ethanol, 75%, according to Roberts *et al.* [18] was used. There was no need to delipidate and desalt the sample by a special procedure since the amounts of GABA were sufficiently high to allow the chromatography of an extract from only about 25 mg. of brain tissue. The non-amino acid components extractable from this amount of brain tissue did not interfere with chromatographic separation.

Paper chromatographic separation. In the solvent system used, GABA was separated from all amino acids except tyrosine; the amount of tyrosine, however, was too low to be detected and therefore could not interfere with the estimation. Alanine and glutamic acid (not fully resolved from threonine) also could be estimated on the chromatogram. In combination with butan-1-ol-formic acid-water (75:15:10, by vol.) all the main free amino acids of rat brain could be estimated, i.e. glutamine, glutamic acid, alanine, GABA and taurine.

Colorimetric estimation. When the ninhydrin-stained chromatograms were drawn through a cupric solution [4, 5] and then the complex extracted with methanol, non-linear and slightly scattered extinction-concentration plots were obtained. This could be explained by the observation that in some denser parts of the spots the red complex was not formed. Addition of cupric salt to the eluting liquid [7], even without previous treatment with copper on the paper, abolished this effect and permitted to obtain smooth standard curves.

Average GABA values. Our results obtained after freezing with liquid nitrogen (Table 1) are in reasonable agreement with those reported by other authors who used liquid oxygen [20, 10, 1]. Liquid nitrogen, which is safer to use than liquid oxygen, has a lower density which facilitates the rapid immersion of the head. The GABA values found in the brain deproteinized, without freezing, immediately after decapitation, are similar to those of Turský & Šajter [22]. Awapara's [3] approximate figures seem to be rather high.

Individual differences. Differences of GABA content in the brain between individual rats were very slight as indicated by the low standard deviation values. The high constancy of GABA content in the brain of animals belonging to the same species has already been noted [17]. If the rapid turn-over of GABA is considered, of which the pronounced *post-*

-*mortem* increase may be a consequence, one must assume a very efficient homeostatic mechanism which keeps the level of this substance within optimal limits. This may serve as an argument in favour of the role of GABA as an indispensable factor in brain function. Yet, as Roberts *et al.* [17, 14] point out, this constancy is not limited to GABA in the brain but applies to other free amino acids and other tissues as well.

In some experiments we have analysed separately the hemispheres, the *rhinencephalon*, *cerebellum* and *medulla oblongata* and only slight differences in GABA concentration were found. These findings remain to be repeated in view of the remarkable differences of GABA content in various areas of the brain reported for other species [3, 13, 17].

The influence of post-mortem temperature. The accuracy of the analytical method and the narrow range of individual variation has permitted us to show that the rapid immersion of the head into a freezing mixture of -75° results in a significantly higher GABA content than that obtained by the liquid nitrogen technique. If freezing is omitted, GABA continues to increase. The fact that the formation of GABA is greater than its degradation is to be expected because the lack of oxygen limits, and finally stops, the oxidation of succinic semialdehyde and succinate, as well as the tricarboxylic acid cycle, whereas the decarboxylation may continue. In addition to decarboxylation, liberation of GABA from a "bound" form must also be considered [21, 19].

The question whether freezing with liquid nitrogen entirely prevents (or at least considerably limits) any *post-mortem* increase in GABA content was investigated by temperature measurements within the brain. It was shown that the time needed for the temperature in the central area of the brain to fall from 38° to 20° was three times shorter in liquid nitrogen than in CO_2 -ethanol; for cooling to lower temperatures, this difference was still greater. If the increase of the free or easily extractable GABA over the zero-time level is proportional to the time during which the enzymic activities continue after the death, then the difference between the (unknown) *in vivo* levels and the levels found after the liquid-nitrogen treatment should be less than half the difference between the levels obtained after the liquid nitrogen and CO_2 -ethanol treatment.

Time-course of the temperature decrease. Even in liquid nitrogen the drop of temperature was sufficiently slow to allow at least 30 sec. of enzymic activity. Therefore it would be useless to attempt to follow by the liquid nitrogen freezing technique any transient biochemical changes in rat brain which might take place in an interval of the order of seconds.

The authors are obliged to Dr. Ivor Smith for the gift of the GABA standard, to Dr. N. B. Myant for reading the manuscript, to Mr. P. Brich for technical assistance in some experiments and to Mr. J. Macků for the thermocouple.

SUMMARY

1. Heads of decapitated rats were immediately immersed (A), in liquid nitrogen or (B), in CO₂-ethanol mixture, or (C), the brain was taken out and deproteinized either immediately or (D), after 30 min. at room temp.; the lowest content of γ -aminobutyric acid was obtained by the liquid nitrogen technique.

2. The course of temp. decrease during freezing of the brain was followed; the decrease was more rapid in liquid nitrogen but even then the enzymic activity could continue for about 30 sec.

REFERENCES

- [1] Ansell G. B. & Richter D. - *Biochem. J.* **57**, 70, 1954.
- [2] Ansell G. B. & Richter D. - *Biochim. Biophys. Acta* **13**, 92, 1954.
- [3] Awapara J., Landua A. J., Fuerst R. & Seale B. - *J. Biol. Chem.* **187**, 35, 1950.
- [4] Bode F., Hübener H. J., Brückner H. & Hoeres K. - *Naturwiss.* **39**, 524, 1952.
- [5] Fischer F. G. & Dörfel H. - *Biochem. Z.* **324**, 544, 1953.
- [6] Florey E. - *Naturwiss.* **44**, 424, 1957.
- [7] Giri K. V., Radhakrishnan A. N. & Vaidyanathan C. S. - *Analyt. Chem.* **24**, 1677, 1952; *J. Indian Inst. Sci.* **33**, 145, 1953.
- [8] Iwama K. & Jasper H. H. - *J. Physiol.* **138**, 365, 1957.
- [9] Killam K. F. - *Fed. Proc.* **17**, 1018, 1958.
- [10] Musaelyan S. S. & Sytinskiĭ I. A. - *Dokl. Akad. Nauk SSSR* **139**, 994, 1961.
- [11] Purpura D. P., Girado M. & Grundfest H. - *Proc. Soc. Exptl. Biol. Med.* **95**, 791, 1957.
- [12] Richter D. & Dawson R. M. C. - *J. Biol. Chem.* **176**, 1199, 1948.
- [13] Roberts E. - *Am. J. Orthopsychiatry* **30**, 15, 1960.
- [14] Roberts E. & Baxter C. F. - *Ann. Rev. Biochem.* **32**, 513, 1963.
- [15] Roberts E. & Frankel S. - *J. Biol. Chem.* **187**, 55, 1950.
- [16] Roberts E., Harman P. J. & Frankel S. - *Proc. Soc. Exptl. Biol. Med.* **78**, 799, 1951.
- [17] Roberts E., Lowe I. P., Guth L. & Jelinek B. - *J. Exptl. Zool.* **138**, 313, 1958.
- [18] Roberts E., Rothstein M. & Baxter C. F. - *Proc. Soc. Exptl. Biol. Med.* **97**, 796, 1958.
- [19] Sano K. & Roberts E. - *Biochem. Biophys. Res. Commun.* **4**, 358, 1961.
- [20] Sotnikova A. P. & Sytinskiĭ I. A. - *Radiobiologiya* **3**, 504, 1963.
- [21] Turský T., private communication.
- [22] Turský T. & Sajter V. - *J. Neurochem.* **9**, 519, 1962.
- [23] Udenfriend S. - *J. Biol. Chem.* **187**, 65, 1950.
- [24] Vladimirov G. E. & Sytinskiĭ I. A. - *Uspekhi Sovrem. Biol.* **51**, 3, 1961.

ZAWARTOŚĆ KWASU γ -AMINOMASŁOWEGO W MÓZGU SZCZURA
PREPAROWANYM W RÓŻNYCH TEMPERATURACH

Streszczenie

1. Odcięte głowy szczurów zanurzano (A), w płynnym azocie, (B), w mieszaninie suchego CO₂ i etanolu; lub (C) mózg izolowano i odbiałczano natychmiast, albo (D) odbiałczano po 30 min. w temperaturze pokojowej. Najniższą zawartość kwasu γ -aminomasłowego otrzymano przy zamrażaniu głowy w płynnym azocie.

2. Badano przebieg spadku temperatury w mózgu podczas zamrażania. Spadek był szybszy przy zastosowaniu płynnego azotu, ale nawet wówczas enzymy mogły działać jeszcze przez ok. 30 sekund.

Received 4 January 1964.

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ON THE NATURE OF PROTEOLYSIS BY CHLOROETHANOLS

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In previous reports [4, 5, 6] we described the proteolytic activity of monochloroethanol with simultaneous partial esterification of the released amino acids. The esterification was confirmed by direct reaction of monochloroethanol with various amino acids carried out under the same conditions as those applied for proteolysis. Also in this case the esterification was not complete; it was the highest with leucine due to the greater stability of this ester. On chromatograms of esterified tri-functional amino acids and glycine we found beside the main ninhydrin-positive spots of the released amino acid and its ester, one or two faint ninhydrin-positive spots. Analysis of a glycine ester hydrochloride preparation revealed a chloroethyl and not an ethyl ester; therefore we assumed that chloroethyl esters are formed also from other amino acids. Dicarboxylic amino acids (Asp, Glu) gave monochloroethyl mono- and diesters as shown by the fact that the acid hydrolysates of the isolated esters gave on paper chromatography only single ninhydrin-positive spots corresponding to the respective amino acids [7, 1].

Proteolysis by trichloroethanol was also demonstrated but of course the esterification was far less effective or was entirely absent (e.g. with glycine) [3].

In the present work the difference between the products of protein decomposition by mono- and trichloroethanol and by hydrochloric acid was investigated.

Chromatography and electrophoresis of the monochloroethanolysates of gelatine, serum albumin and globulin revealed more ninhydrin-positive spots as compared with acid hydrolysates (Fig. 1 and 2) but, unlike in acid hydrolysates, no acidic amino acids were found. This can be explained by their esterification and eventually by decarboxylation, but this problem has not yet been investigated.

An attempt was also made to elucidate the mechanism of chloroethanolysis. In the experiments reported previously [5, 6] the influence of water was not excluded. Although proteins were dried *in vacuo* over

phosphorus pentoxide, still some firmly bound water may have been present [8, 2]. Dehydrated monochloroethanol was used, but it is a very hygroscopic compound. Furthermore, water could be formed in the esterification reaction of the free protein carboxyl groups or during the formation of humins from some amino acids.

In the monochloroethanolysate of a dried protein, 4.1% of chloride ion was found both by mercurimetric and polarographic methods; this value may be assumed to correspond to the hydrochloride split from monochloroethanol. When proteolysis was carried out with chloroethanol

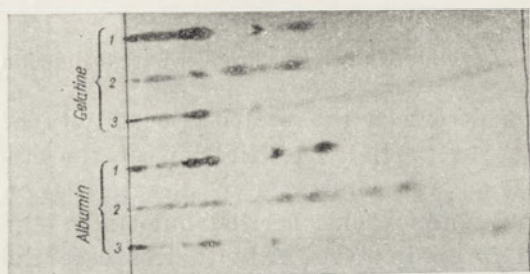
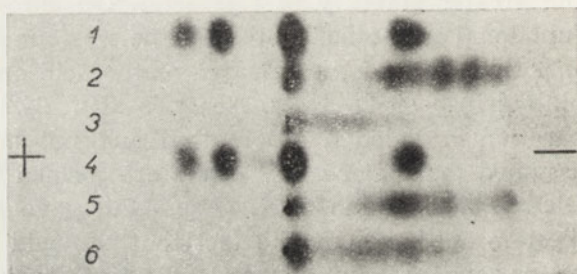


Fig. 1. Chromatograms of gelatine and serum albumin: (1), acid hydrolysate; (2), monochloroethanolysate; (3), trichloroethanolysate.

Fig. 2. Electrophorograms of gelatine: (1), acid hydrolysate; (2), monochloroethanolysate; (3), trichloroethanolysate; and of serum globulin: (4), acid hydrolysate; (5), monochloroethanolysate; (6), trichloroethanolysate.



containing 10% of water, the amount of chloride ion was doubled. To eliminate the effect of the hydrochloride, chloroethanol proteolysis was performed with an addition of calcium carbonate or magnesium oxide.

In the experiments with calcium carbonate, proteolysis and especially esterification and humin formation were markedly lower, as was shown by very faint ninhydrin-positive spots. As the reaction $\text{CaCO}_3 + 2 \text{HCl} \rightleftharpoons \text{CaCl}_2 + \text{H}_2\text{CO}_3$ took place in a sealed tube, the presence and action of hydrogen ions could not be completely excluded.

To eliminate the effect of hydrogen ions and water, the reaction mixture of protein and chloroethanol was added with magnesium oxide, which is able to bind both water and hydrochloride. This resulted in a nearly complete suppression of proteolysis by monochloroethanol or in the case of trichloroethanol even in disappearance of the ninhydrin-positive spots. At the same time the formation of humin was increased and as we do not know its derivation, this interferes with the inter-

pretation of the test. If chloroethyl ester was formed in the neutral solution, then we can assume that, contrary to its hydrochloride, it underwent cyclization. To check this supposition, experiments with chloroethyl ester of glycine hydrochloride are being performed. It seems that the presented results permit to reject transesterification as the only mechanism of this particular proteolysis; however, they indicate that hydrogen ions have an activating effect on proteolysis by chloroethanol.

MATERIALS AND METHODS

Proteins: gelatine Difco for bacteriological purposes, and serum albumin and serum globulin prepared by ethanol fractionation, were used.

2-Chloroethanol, Lachema, if not practically neutral (pH 6), was freed from acid and water and then distilled. Only a quite colourless neutral fraction, b.p. 126° - 127° , was used.

2,2,2-Trichloroethanol (Light & Co.), supplied as a brownish liquid, was submitted to fractional distillation under reduced pressure (20 mm. Hg) and eleven fractions were obtained. Fractions 3 - 10, which immediately after distillation were colourless, later turned brownish till brown-violet. As their boiling points (57° - 60° /20 mm.Hg) and refractive indices (1.4875 - 1.4895) were fairly close, these fractions were combined. They were first shaken with active charcoal, then rapidly distilled *in vacuo*. The product was quite colourless and its refractive index was 1.4890.

For experiments, samples (mostly of 100 mg.) of proteins or amino acids were mixed with 20-fold volume of, respectively, 6 N-HCl, or mono- or trichloroethanol and then heated in sealed tubes at 100° - 105° for 100 hr. Then the tubes were opened and aliquots were spotted on paper.

Paper chromatography was carried out by the descending technique in the Patridge system of *n*-butan-1-ol - acetic acid - water (4:1:5, by vol.). Chromatograms were dipped into 2% ninhydrin solution in acetone and colour stabilized with cupric nitrate.

Electrophoresis was carried out on Whatman no. 3 paper (14×46 cm.) in a buffer of pyridine - acetic acid - water (1:1:45, by vol.) at pH 5.1, 300 V, 30 - 35 mA, for 4 hr. Detection of the spots was made in the same way as for chromatography.

Water eluates of esters were evaporated in a desiccator over potassium hydroxide; the dry residue was dissolved in a small amount of 6 N-HCl and hydrolysed for 12 hr. in a sealed capillary tube. The hydrolysate was dried as above, the residue dissolved in a drop of water and submitted to paper chromatography and electrophoresis.

SUMMARY

The previously reported proteolysis by mono- and trichloroethanol was compared with acid hydrolysis. Paper chromatography and electrophoresis revealed differences in the obtained products. The proteolysis by chloroethanol was inhibited by the addition of calcium carbonate or magnesium oxide. Therefore it is assumed that this particular proteolysis is activated by hydrogen ions.

REFERENCES

- [1] Duchoň J., Čejková B. & Richter A. F. - *Acta Univ. Carol., Medica*, Suppl. **14**, 257, 1961.
- [2] Lloyd D. J. & Moran T. - *Proc. Roy. Soc. A, London* **147**, 382, 1934.
- [3] Richter A. F., Čejková B. & Duchoň J. - *Acta Univ. Carol. Med. Suppl.* **14**, 265, 1961.
- [4] Richter A. F. & Duchoň J. - *Lékařské listy*, **9**, 33, 1954.
- [5] Richter A. F. & Duchoň J. - *Čas. lék. čes.* **97**, 229, 1958.
- [6] Richter A. F. & Duchoň J. - *Acta Univ. Carol., Medica* **413**, 1958.
- [7] Richter A. F. & Duchoň J. - *Čas. lék. čes.* **100**, 425, 1961.
- [8] Sary Z. - *Z. physiol. Chem.* **186**, 137, 1930.

O PROTEOLIZIE WYWOŁANEJ PRZEZ CHLOROETHANOL

Streszczenie

Opisaną poprzednio proteolizę wywołaną mono- i trójchloroetanolem porównano z hydrolizą kwaśną. W produktach reakcji wykazano różnice chromatograficznie i elektroforetycznie. Dodanie węglanu wapnia lub tlenku magnezu hamuje proteolizę chloroetanolem. Można przyjąć, że tę specjalną proteolizę aktywują jony wodoru.

Received 4 January 1964.

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**ON THE USE OF ^{32}P -LABELLED CYTIDINE DIPHOSPHATE BASES
FOR THE *IN VITRO* STUDIES ON THE METHYLATION
OF PHOSPHOLIPIDS WITH S-ADENOSYLMETHIONINE**

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The possibility of obtaining several analogues of phospholipids labelled with ^{32}P from the respective cytidine diphosphate bases [1, 2, 8, 7] provides a new convenient approach for studying the process of methylation of phospholipids. A very simple procedure for separation of phospholipids on aluminum oxide column allows to follow the course of methylation by studying the intact phospholipids. This simplifies greatly the studies, as they can be performed by using also a ^{32}P label. The aim of this paper was to examine the adequacy of this approach for studying the formation of lecithin *via* the methyl group transfer onto phospholipid acceptors. Both the biosynthesis of several analogues of phosphatidylcholine and the use of aluminum oxide for their chromatographic separation have been previously reported [1, 2, 8, 7]. Some of the data of the present paper have been included in a preliminary communication [9].

EXPERIMENTAL AND DISCUSSION

Special reagents. Dimethylaminoethanol (DMAE)¹ and monomethyl aminoethanol (MMAE), L. Light & Co., Colnbrook, England; dicyclohexylcarbodiimide, BDH, London; CMP, Sigma, U.S.A.; DL-methionine, Fluka, Switzerland. The unlabelled O-phosphoryl esters of MMAE and DMAE were prepared and kindly provided by Dr. G. B. Ansell from Birmingham University, England. Aluminum oxide, Brockman activity 2, was Savory & Moore, Great Britain, product. [^{32}P]Orthophosphate was of French origin.

[^{32}P]O-Phosphoryl esters of monomethylaminoethanol and dimethylaminoethanol were prepared by heating at about 140° equimolar amounts

¹ Abbreviations used: CMP, cytidine-5'-phosphate; E, ethanolamine; MMAE, N-monomethylaminoethanol; DMAE, N,N-dimethylaminoethanol. The prefix P indicates the phosphoryl esters of the bases; CMP-, cytidine derivatives.

of the base and [^{32}P]orthophosphoric acid under reduced pressure during 6 hr. over P_2O_5 . For preparing phosphorylethanolamine the mixture of ethanolamine and [^{32}P]orthophosphoric acid was heated at about 180° for 3 hr. [4]. ^{32}PE and $^{32}\text{PMMAE}$ were isolated from the reaction mixture, after dilution with water, hydrolysis on a boiling water bath and adjusting to pH 8 with 0.5 N-NaOH, by passing it through a short column of Dowex 1 formate. After washing the column with water the phosphoryl esters were eluted with 0.05 N- HCOOH . For isolation of $^{32}\text{PDMAE}$ the hydrolysed reaction mixture was passed through a column of Amberlite IR 120 (NH_4^+ form), the effluent adjusted to pH 8 and passed through a column of Amberlite IRA 400 (Cl^- form). If in the effluent some orthophosphate was still present, the passage through the Amberlite IRA 400 (Cl^- form) was repeated.

The synthesis of CMP- ^{32}PE , CMP- $^{32}\text{PMMAE}$ and CMP- $^{32}\text{PDMAE}$ was performed from the respective O-phosphoryl esters and CMP according to Kennedy [15]. For isolation of CMP- ^{32}PE and CMP- $^{32}\text{PMMAE}$ the reaction mixtures were first subjected to paper chromatography as described previously [7]. From the chromatogram, the radioactive and UV absorbing band was cut off, washed with ether, eluted with water and the eluate chromatographed on Dowex 1 formate column [15] as described previously [8]. CMP- $^{32}\text{PDMAE}$ was isolated by column chromatographic procedure. The molar ratios cytidine:phosphorus of the labelled nucleotides (calc. 1:2) were: CMP-PE, 1:1.89; CMP-PMMAE, 1:1.95, and CMP-PDMAE, 1:1.92, and the specific activities were 50 000, 72 400 and 44 600 counts/min./ μmole , respectively.

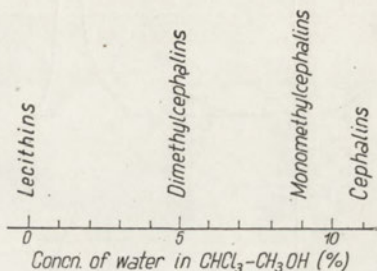
S-Adenosylmethionine was obtained (1.2 g.) from a culture of commercial baker yeast (250 g.) grown for 32 hr. with addition of 2 g. of DL-methionine according to Schlenk & DePalma [20]. The preparation exhibited the molar ratio of adenine to ribose 1:1.04 (calc. 1:1). It was homogeneous when tested by paper chromatography: R_f 0.28 in ethanol-water-acetic acid (65:34:1, by vol.) on Whatman no. 1 paper. On acid hydrolysis (1 hr., 1 N-HCl, 100°) it was completely decomposed with formation of methionine.

Analytical methods. The content of ribose was estimated by the orcinol method [17] and that of adenine was determined spectrophotometrically on the basis of m-molar extinction coefficient of 15.0 at 256 m μ in acid [20]. The details of estimation of phosphorus, cytidine and radioactivity were the same as previously described [7].

Incubation of tissue homogenates. The experiments were performed with rat liver. The tissue homogenates were incubated for 30 min. with the labelled cytidine diphosphate aminoethanols, then S-adenosylmethionine was added to the incubate and the incubation continued for 30 min. The details of incubation and extraction of lipids were the same as previously described [7].

Chromatography of phospholipids on aluminum oxide columns. The evaporated lipid extract from the incubated sample (about 100 μ g. of lipid phosphorus) was dissolved in 5 ml. of chloroform and fractionated on a 3×0.7 cm. aluminum oxide column. The upper part of the column was fitted with a reservoir, 2 cm. in diameter, to lower the hydrostatic pressure of the eluting mixture and thus to slow down the rate of the elution. The elution was carried out with 3 ml. portions of chloroform-methanol (1:1, v/v) containing increasing concentrations, up to 14%, of water. With no water added, the choline lipids were eluted [2]. Other phospholipids were eluted as follows: phosphatidyl dimethylaminoethanol at 5% concentration of water, phosphatidyl monomethylaminoethanol at 9%, and phosphatidylethanolamine at 12% (Fig. 1). The whole

Fig. 1. Diagram of the elution of methylcephalins from aluminum oxide column with chloroform-methanol (1:1, v/v) mixtures containing increasing concentrations of water.



procedure of the elution can be completed within 15-20 min. No more radioactivity could be eluted by further increasing the concentration of water in the chloroform-methanol mixture, but on changing the solvent to chloroform-ethanol-water (2:5:2, by vol.) [11] some additional 30% of radioactive lipid formed from CMP- ^{32}PE was recovered. This was only 20% in the case of phospholipids formed from CMP- $^{32}\text{PMMAE}$ and none was found when the radioactive phospholipid was derived from CMP- $^{32}\text{PDMAE}$ or cytidine diphosphate choline. The more tightly bound fractions have not been further studied.

The method applied was originally devised by Long & Staples [16] for separating cerebrosides from cephalins. In our experience, the separation of methylcephalins from tissue extracts on silicic acid column [14] seemed less encouraging, as no satisfactory separation of DMAE-phospholipid from lecithin was obtained on increasing methanol concentration in chloroform. The Dawson's method [12] using two-dimensional chromatography of the products of mild alkaline hydrolysis, also could not be applied because the separation from glycerylphosphorylcholine and glycerylphosphorylethanolamine was unsatisfactory. Other methods for detection of methylcephalins based on complete hydrolysis of the lipid sample and estimation of free dimethyl- and monomethylaminoethanol by the distillation procedure [3] or chromatography on Dowex 50 resin [18], are not appropriate for studies with ^{32}P .

The elution patterns of phospholipids formed in the homogenates from CMP-³²PE, CMP-³²PMMAE and CMP-³²PDMAE (Fig. 2, part I) indicate that the radioactivity is found in the fractions of a respective cephalin. It can thus be concluded that during the incubation no methylation or demethylation has occurred to any appreciable extent. On the other hand, the addition of *S*-adenosylmethionine caused the conversion

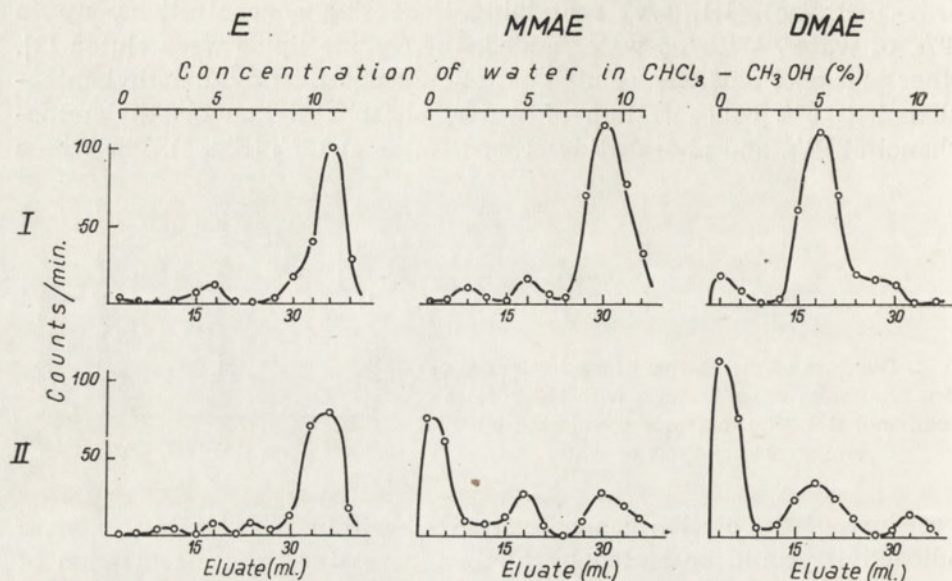


Fig. 2. Chromatography on aluminum oxide columns of phospholipids formed from CMP-³²PE, CMP-³²PMMAE and CMP-³²PDMAE in tissue homogenates of rat liver without (I) and with (II) the addition of *S*-adenosylmethionine. The incubation of 100 mg. of homogenized tissue was performed in the medium containing: 31.6 mM-KCl; 9.5 mM-NaF; 20 mM-MgCl₂; 4 mM-Na₂HPO₄; 26.6 mM-tris-HCl buffer, pH 7.4, and the respective cytidine nucleotides (0.05 μ mole) as indicated: E, CMP-³²PE; MMAE, CMP-³²PMMAE; DMAE, CMP-³²PDMAE in the final volume of 1.5 ml. at 37° for 1 hr. To the samples (II) 2 μ moles of *S*-adenosylmethionine were added after the first 30 min. of incubation. The lipids were extracted according to Folch *et al.* [13] and chromatographed on 3 \times 0.7 cm. aluminum oxide columns with 3 ml. portions of chloroform-methanol (1:1, v/v) mixtures containing increasing concentrations of water as indicated in the top part of the Figure.

of phosphatidyl dimethylaminoethanol into lecithin, eluted in the first 6 ml. of the effluent, and of MMAE-phospholipid into DMAE-phospholipid and lecithin (Fig. 2, part II). It is rather improbable that the methylation could concern the cytidine coenzyme, which may also lead to the formation of lecithin, because after 30 min. of incubation, i.e. at the time when *S*-adenosylmethionine was added, already no cytidine coenzyme was present. A part of the amount added (about 20%) gave rise to the phospholipid and the remainder was decomposed as it was proved by

following the disappearance of the nucleotide by absorption on charcoal. It has been reported by Bremer & Greenberg [5] and Gibson *et al.* [14] that *S*-adenosylmethionine methylates the phospholipids, but not water-soluble forms of aminoethanols, i.e. aminoethanol phosphate or its cytidyl derivative. The methylation of methylcephalins was incomplete as can be seen from Fig. 2; the phospholipids originally formed from CMP-³²PMAE and CMP-³²PDMAE, were still present despite the molar ratio of the newly synthesized phospholipids to *S*-adenosylmethionine added, being about 1:200. No conversion of cephalin formed from CMP-³²PE was observed upon addition of *S*-adenosylmethionine to the incubate. This seems to agree with the difficulty of introducing the first methyl group into cephalin, reported also by other authors [6, 10], who claimed that this was the rate-limiting step in the whole methylation process leading to lecithin. There is also an indication that another one-carbon-group donor but not *S*-adenosylmethionine is involved in the formation of phosphatidylmonomethylaminoethanol [21]. It could also be that the fatty acid composition of cephalin formed from CMP-³²PE was responsible for the lack of methylation. Bremer & Greenberg [6] using ¹⁴C-labelled methyl group donor have shown no methylation of dipalmitoylcephalin while distearoylcephalin was a good acceptor of methionine methyl group. The ³²P-labelled cephalin was used by Popják & Muir [19] for the *in vivo* studies on the formation of lecithin, however, the nature of the methylating agent and the fatty acid composition of phospholipids were not considered. It was also recently found [9] that phospholipids formed from cytidine diphosphate 3-dimethylaminopropan-1-ol and other unnatural bases are not methylated by *S*-adenosylmethionine, pointing to the specificity of methyltransferase toward phospholipids with a definite fatty acid and base composition.

It can be supposed that both processes of formation of lecithin, from cytidine diphosphate choline and *via* the methylation of mono- and dimethylcephalins, may occur. The transfer of two methyl groups onto methylcephalin seems to be a two-step reaction; the intermediate product, dimethylcephalin, was present beside lecithin although in a smaller amount. This finding is at variance with the calculation of Cooksey & Greenberg [10] who suggested on the basis of experiments with *S*-adenosyl-[Me-¹⁴C]methionine that once a methylcephalin is formed, it does not undergo further methylation. The authors pointed at the necessity of using [³²P]phosphatidylaminoethanols to elucidate this problem.

It is hoped that the preparation of labelled phospholipids from synthetic cytidine precursors together with the applied procedure of chromatography on aluminum oxide will provide an useful tool in studies on methylation of phospholipids.

The authors have pleasure to acknowledge the co-operation of Dr. G. B. Ansell in performing these experiments and thank him for providing some chemicals.

SUMMARY

The formation of lecithin from *S*-adenosylmethionine and ^{32}P -labelled mono- and dimethylcephalins formed from cytidine diphosphate mono-methylaminoethanol and cytidine diphosphate dimethylaminoethanol in tissue homogenates of rat liver was demonstrated. The course of the methylation of phospholipids was studied by the column chromatographic procedure on aluminum oxide.

REFERENCES

- [1] Ansell G. B. & Chojnacki T. - *Biochem. J.* **85**, 31P, 1962.
- [2] Ansell G. B. & Chojnacki T. - *Nature* **196**, 545, 1962.
- [3] Artom C., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 358, Academic Press, New York, 1957.
- [4] Artom C., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 4, p. 815, Academic Press, New York, 1957.
- [5] Bremer J. & Greenberg D. M. - *Biochim. Biophys. Acta* **37**, 173, 1960.
- [6] Bremer J. & Greenberg D. M. - *Biochim. Biophys. Acta* **46**, 205, 1961.
- [7] Chojnacki T. - *Acta Biochim. Polon.* **11**, 11, 1964.
- [8] Chojnacki T. & Korzybski T. - *Acta Biochim. Polon.* **10**, 233, 1963.
- [9] Chojnacki T., Korzybski T. & Ansell G. B. - *Biochem. J.* **90**, 18P, 1964.
- [10] Cooksey K. E. & Greenberg D. M. - *Biochem. Biophys. Res. Comm.* **6**, 256, 1961.
- [11] Davison A. N. & Wajda M. - *Biochem. J.* **82**, 113, 1962.
- [12] Dawson R. M. C. - *Biochim. Biophys. Acta* **14**, 374, 1954.
- [13] Folch J., Lees M. & Sloane-Stanley G. H. - *J. Biol. Chem.* **226**, 497, 1957.
- [14] Gibson K. D., Wilson J. D. & Udenfriend S. - *J. Biol. Chem.* **236**, 673, 1961.
- [15] Kennedy E. P. - *J. Biol. Chem.* **222**, 185, 1956.
- [16] Long C. & Staples D. A. - *Biochem. J.* **80**, 557, 1961.
- [17] Mejbbaum W. - *Z. physiol. Chem.* **258**, 117, 1939.
- [18] Pilgeram L. O., Gall E. M., Sassenrath E. N. & Greenberg D. M. - *J. Biol. Chem.* **204**, 367, 1953.
- [19] Popják G. & Muir H. - *Biochem. J.* **46**, 103, 1949.
- [20] Schlenk F. & DePalma R. E. - *J. Biol. Chem.* **229**, 1051, 1957.
- [21] Stekol J. A., Weiss S. & Anderson E. J. - *J. Am. Chem. Soc.* **77**, 5192, 1955.
- [22] Wilgram G. F. & Kennedy E. P. - *J. Biol. Chem.* **238**, 2615, 1963.

ZASTOSOWANIE ZNAKOWANYCH ^{32}P CYTYDYNODWUFOSFOZASAD JAKO PREKURSÓRÓW FOSFOLIPIDÓW DO BADAŃ IN VITRO NAD METYLACJĄ FOSFOLIPIDÓW PRZEZ *S*-ADENOZYLOMETIONINE

Streszczenie

Znakowane ^{32}P mono- i dwumetylokefaliny wytworzone w homogenacie wątroby szczura z cytydynodwufosfomonometyloaminoetanolu i cytydynodwufosfodwumetyloaminoetanolu ulegają w obecności *S*-adenozylometioniny przekształceniu w lecytynę. Rozdział fosfolipidów ulegających metylowaniu przeprowadzono na kolumnach tlenku glinu.

Received 4 January 1964.

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STUDIES ON THE IMIDAZOLYTIC AND EXCHANGE REACTIONS OF NADase FROM CARP LIVER MITOCHONDRIA

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Kaplan, Zatman *et al.* [17, 11, 12] postulated both hydrolytic and transglycosidic function of NADases in animal tissues. This is at variance with our previous results [16] which showed that NADase from carp liver mitochondria is "insensitive" to isonicotinyl hydrazide (INH), but does not exhibit transglycosidic action towards this compound. The aim of the present work was therefore to examine pyridine derivatives other than INH as substrates for carp NADase in the transglycosidase reaction. For fuller characterization of the carp enzyme the "imidazolytic" cleavage of NAD, described by Alivisatos *et al.* [2] was studied.

EXPERIMENTAL

Materials and Methods

Enzymic preparations. Homogenates and extracts from mitochondrial and nuclear fractions of liver from carp, lizard and frog (*Rana esculenta*) were prepared in the same way as previously described [16].

NADase from the extract of carp liver mitochondria was purified about 40-fold after Abdel-Latif & Alivisatos [1] on the calcium phosphate gel-DEAE cellulose column at 4°. The fraction eluted with 0.1 M-potassium phosphate buffer, pH 7.1, was collected; the specific activity was 400 units/mg. of protein. NADase from beef spleen was extracted with 0.02 M-NaHCO₃ and purified according to Kaplan [10] to contain 30 units/ml.

Reagents. INH analogue of NAD (INH-adenine dinucleotide) was synthesized after Zatman *et al.* [19]. NAD (C. F. Boehringer & Soehne, Mannheim, Germany), 3-acetyl pyridine, pyridine-3-aldehyde, methyl isonicotinate, and ethylnicotinate were a gift from Prof. Dr. Halina Dahling from the Pharmaceutical Institute, Warsaw, and isonicotinyl hydrazide (INH) was a gift from the Department of Organic Technology of the Warsaw Politechnic School. Histamine·HCl (Schuchardt, Munich,

Germany); histamine, and ethylamine (Fluka, Switzerland); sodium-pyrophosphate (Hurt. Farm., Katowice, Poland); tannin (Ce-Farm, Gliwice, Poland); crystalline yeast alcohol dehydrogenase (Nutritional Biochemical Corp., U.S.A.); DEAE-cellulose (Whatman Batch No. 542-550); calcium phosphate gel was prepared after Colowick [7]; urocanic acid (CalBiochem., U.S.A.); orcinol (E. Merck, U.S.A.), was recrystallized.

Preparation of adenosine diphosphate ribose (APPR). This was prepared after Kaplan *et al.* [14]; 0.1 g. of NAD dissolved in 0.05 M-phosphate buffer, pH 7.6, was treated with 240 units of carp liver NADase at 37°. The reaction was completed within 2 hr. The mixture was deproteinized with 10% TCA (1:1) in cold and the APPR was precipitated with 5 volumes of acetone; the precipitate was washed twice with alcohol-ether mixture (4:1 and 1:1) and ether. The yield of crystalline APPR was about 30%. All the operations were carried out at -10°.

The enzyme assay. Determination of the NADase activity was based on spectrophotometric measurements of the decrease in extinction at 325 m μ of NAD-KCN complex and of NAD at 340 m μ with the yeast alcohol dehydrogenase [10] as described previously [16]. The effect of INH, methylisonicotinate, ethylnicotinate and 3-acetylpyridine on the purified NADase preparation from carp liver mitochondria was examined in concentration 0.01 M after a 10 min. incubation period at 37°. A unit of NADase was defined according to Kaplan [10] as that amount which cleaves 1 μ mole of NAD per hour. Calculation was based on activity measurement after a 10 min. incubation period [16].

Estimation of NAD-analogues. INH, ethylnicotinate, 3-acetylpyridine and pyridine-3-aldehyde, in concentration 0.1 M were incubated with 5 μ moles of NAD, and the purified carp liver NADase (5 units) in phosphate buffer, pH 7.6, in total volume of 3 ml. The 0.1 ml. samples were withdrawn at various time intervals. Formation of INH-analogue of NAD was measured by the change of extinction at 385 m μ after addition of 0.1 M-NaOH. The acetylpyridine and pyridine-3-aldehyde analogues of NAD function as dehydrogenase coenzymes, therefore the formation of these analogues was estimated spectrophotometrically after enzymic reduction at 365 and 400 and at 355 m μ , resp. [11, 13]. Formation of the ethylnicotinate analogue of NAD was measured on the basis of the extinction of its complex with KCN and lack of reduction by alcohol dehydrogenase [13].

Determination of protein. The protein in the extracts from liver mitochondria and nuclei was measured by the tannin method of Mejbaum-Katzenellenbogen [15] and in the mitochondrial and nuclear homogenates by the Kjeldahl method.

Estimation of the histamine derivative of APPR. The formation of this derivative during incubation was followed by measuring the decrease in NAD concentration by the cyanide method, and that of

ribose in NAD by the orcinol method of Mejbaum as modified by Horecker *et al.* [9]. The decrease in ribose as determined by this method is due to the formation of histamine-ribose bond resistant to acid hydrolysis [3, 6]. The incubation mixture contained in total volume of 3 ml.: NADase, alternatively from carp liver mitochondria or beef spleen (3 units), 1.4 μ mole of NAD or 3 μ moles of APPR, 4.5 m-moles of histamine in 0.1 M-Na pyrophosphate buffer, pH 8.3. The controls were run with the enzyme or the nucleotide substrate omitted. The 0.1 ml. aliquots were withdrawn for ribose determination and the 1 ml. aliquots for NAD measurements. The reaction mixture was deproteinized with 80% TCA and separated by the ascending paper chromatography on Whatman no. 1 paper in 0.1 M-acetic acid - ethanol (1:1) system [6]. Histamine-adenine dinucleotide, NAD and APPR were located under UV light and by the perchlorate ammonium molybdic test of Hanes & Isherwood [8] for nucleotides. The non-enzymic formation of N-ribosyl bonds resistant to acid hydrolysis in the orcinol method was followed in the systems containing 1.5 m-moles histamine, 1.5 m-moles ethylamine or 0.25 m-moles urocanic acid, and alternatively 1 μ mole APPR or 2 μ moles ribose, in 1 ml. of 0.1 M-pyrophosphate buffer, pH 8.3, at 37°.

RESULTS

Transglycosidic action of NADases. The purified NADase (400 units/mg. of protein) from carp liver mitochondria was found to be insensitive to INH, 3-acetylpyridine, 3-pyridine-3-aldehyde, methylisonicotinate and ethylnicotinate in concn. 10^{-2} M and did not form the corresponding NAD-analogues as found by the spectrophotometric and enzymic techniques. The purified enzyme was inactive towards the INH analogue of NAD, obtained by the use of the pig brain NADase. These results confirm those obtained previously with the extract of carp liver mitochondria [16] and INH. Nor did the purified enzyme catalyse the exchange reactions with the pyridine derivatives which serve as efficient substrates in the analogous reaction with the INH-insensitive NADases from the avian and mammalian tissues.

To see whether the carp enzyme is an exception among the animal NADases the experiments were performed with the lizard and frog liver.

The activity of NADase in the extract of lizard liver mitochondria was even higher than that observed with the carp enzyme and amounted to 190 units/mg. of protein. The enzyme, similarly to the carp liver NADase, was insensitive to 10^{-2} M-INH and did not form an INH-analogue of NAD. The NADase activity could not be detected in the extract of mitochondria and nuclei of frog liver. Traces of NAD-splitting activity

(0.03 units/mg. of protein) could be detected in the mitochondrial homogenates indicating firm bounding of this enzyme with the cell structures.

Formation of a histamine derivative of APPR. Formation of the histamine derivative of APPR was proved on incubating NAD with the carp NADase in 1.0 - 1.5 M-histamine at pH 8.3 at 37° and determining the decrease in ribose content measurable by the modified orcinol method. No decrease was observed when histamine was incubated with NAD without the enzyme. The decrease in ribose content within 3 hr., due to the formation of a ribose-histamine bond resistant to acid hydrolysis, amounted to about 40% which indicated formation of the histamine derivative of APPR with 80% yield. The same rate of the reaction was obtained with the purified NADase from beef spleen (Fig. 1).

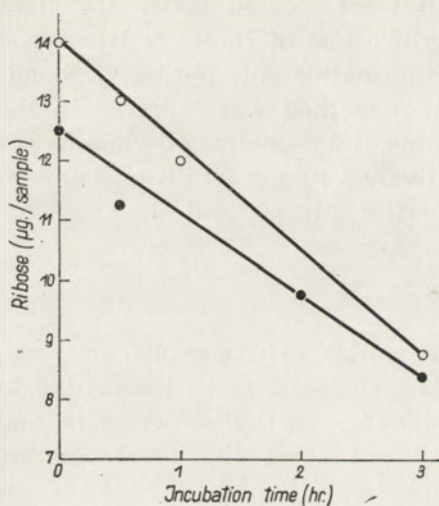


Fig. 1

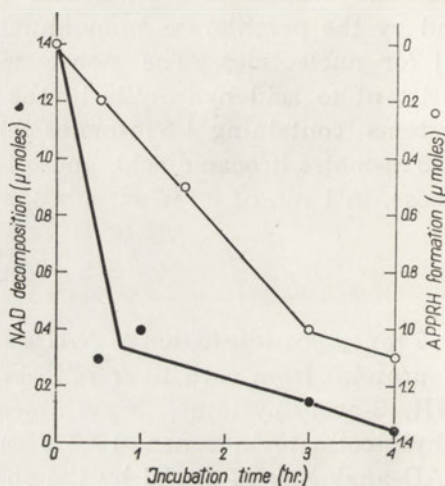


Fig. 2

Fig. 1. Formation of the histamine derivative of APPR expressed as a decrease of ribose. Incubation mixture contained in 3 ml., the purified NADase (3 units), 1.4 μmoles of NAD, 3 m-moles of histamine and 0.1 M-pyrophosphate buffer, pH 8.3. Ribose was in 0.1 ml. samples determined by the orcinol method. (O), NADase from carp liver mitochondria, (●), NADase from beef spleen.

Fig. 2. The relationship between APPRH formation and NAD decomposition in the NAD - histamine - NADase system at pH 8.3. Details see Table 1 and Methods. (●), NAD; (O), APPRH.

Formation of a histamine derivative of APPR was accompanied by the appearance of a spot (R_F 0.46) in the ethanol-0.1 M-acetic acid system, detected under UV and by the molybdcic test. This spot corresponds to that of an histamine-adenine dinucleotide studied by Alivisatos *et al.* [6]. Simultaneously the spot corresponding to APPR (R_F 0.66) was observed on the chromatograms.

Determination of NAD and ribose during incubation of NAD with histamine in the presence of carp NADase (Table 1 and Fig. 2) did not

show proportional relationship between NAD decomposition and formation of the histamine derivative of APPR. Formation of the ribose-histamine bond was preceded by the accumulation of APPR resulting from the hydrolytic decomposition of NAD by NADase. This suggests that this formation is not an "imidazolytic" cleavage of the type postulated by Alivisatos [2], but it is rather a two-step reaction involving APPR as an intermediate.

Table 1

Formation of the histamine derivative of APPR by the NADase from carp liver mitochondria

The composition of the incubation mixture as described under Methods. NAD was determined by the cyanide method; the histamine derivative of APPR (APPRH) was calculated from the decrease in ribose; APPR was calculated from the difference between NAD decrease and APPRH formation.

NAD determined at zero time by the cyanide method and calculated from the ribose content was 1.4.

Incubation time (min.)	NAD (μ moles)		APPRH formed (μ moles)	APPR formed calculated (μ moles)
	found	decrease		
Experiment 1				
0	1.4		0	0
30	0.30	1.1	0.20	0.90
120	0.15	1.25	1.0	0.25
Experiment 2				
0	1.4		0	0
60	0.40	1.0	0.50	0.50
180	0.01	1.39	1.10	0.29

To test this hypothesis APPR was prepared and used as a substrate for the carp NADase in 1.5M-histamine. The results presented in Fig. 3A show that the decrease of estimated ribose characteristic for the formation of the histamine-ribose bond was the same with and without the enzyme. In both cases about 25% of ribose was bound in the ribose-histamine bond resistant to acid hydrolysis. Similar phenomena were observed (Fig. 3B) upon incubating ribose in 1.5M-histamine or APPR in 1.5M-ethylamine at pH 8.3 at 37°. In the first case the decrease in ribose content was 50% after 4 hr. which indicates total conversion of APPR to its histamine derivative. In the second case the decrease was 30% which corresponds to about 60% of ribose bound in the ethylamine derivative of APPR. No changes in ribose content were, however, observed when APPR was incubated with 0.25M-urocanic acid.

These results indicate that formation of the histamine derivative of APPR from NAD in the presence of the carp NADase resulted from

non-enzymic reaction of histamine with APPR formed by the enzyme. It seems, however, that the reaction involved amino-N rather than imidazole-N since ethylamine and not urocanic acid was effective in the

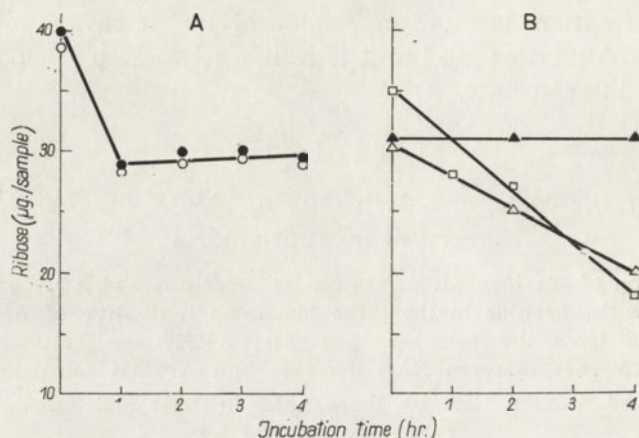


Fig. 3 The non-enzymic formation of the acid-resistant N-ribosyl bonds. A. The incubation mixture contained in 1 ml., 1.3 μ moles of APPR and 1.5 m-moles of histamine in 0.1 M-Na pyrophosphate buffer, pH 8.3. B. The incubation mixture contained in 1 ml., 1 μ mole of APPR or 2.3 μ moles of ribose and 1.5 m-moles of histamine, 1.5 m-moles of ethylamine or 0.25 m-moles of urocanic acid alternatively, in 0.1 M-Na pyrophosphate buffer, pH 8.3. (●), APPR, histamine and the enzyme; (○), APPR and histamine; the enzyme omitted; (□), ribose and histamine; (Δ), APPR and ethylamine; (▲), APPR and urocanic acid.

reaction. Hence the histamine derivative of APPR obtained by us despite the same R_F in ethanol-0.1 M-acetic acid system seems to be a different compound from the histamine-adenine dinucleotide, a product of an imidazolytic cleavage of NAD, obtained by Alivisatos *et al.* [6].

DISCUSSION

NADases in animal tissues were reported by Zatman, Kaplan, Colowick & Ciotti [17, 18, 19] to possess both transglycosidic and hydrolytic properties, as they catalyse formation of various NAD-analogues in reversible exchange reactions. On the other hand, Alivisatos *et al.* [2-6] found that the imidazole derivatives can compete with water for the NAD-enzyme complex in the irreversible reaction similar to hydrolysis in which quaternary nitrogen structure is converted to a trivalent one with the concomitant liberation of H^+ . Alivisatos claimed that this type of NAD breaking involves imino-N of imidazole ring. Participation of the amino group was excluded since the imidazole benzimidazole and 5,6-dimethylimidazole dinucleotides were synthesized by the beef spleen NADase from the corresponding bases and NAD [5]. Histidine was a very poor substrate in this reaction [4].

A highly active NADase from carp liver mitochondria catalysed neither the Kaplan's exchange reaction nor an imidazolytic process of Alivisatos proving thus high affinity of the NAD-enzyme complex for water molecules.

Similarly to the carp enzyme, NADase from the lizard liver mitochondria is only a glycohydrolase with no transglycosidase properties. No information is available concerning properties of NADases from bacteria, invertebrates or lower vertebrates. The NADase from *Neurospora crassa* was formerly [17] reported to be an INH and nicotinamide insensitive enzyme which does not form any pyridine analogues of NAD. The only NADases known so far that catalyse the exchange reaction with the pyridine derivatives are those derived from the avian and mammalian tissues. This indicates that double function can not be assigned to the NADases from all the animal tissues. It seems also that the hydrolytic property is rather a primary, not a secondary phenomenon.

The "imidazolytic" process was studied only with the beef spleen enzyme and carp enzyme, therefore no general conclusions can be drawn about the specificity of the NADase protein for the imidazole substrates.

Attention should be drawn to the acid-resistance of N-ribosyl bond in the histamine derivative of APPR formed non-enzymically from APPR. The reaction observed involving amino group of histamine and the aldehyde group of the sugar may represent the reaction of the Maillard type.

SUMMARY

1. The purified NADase from carp liver mitochondria is "insensitive" to INH, ethylnicotinate, 3-acetylpyridine, pyridine-3-aldehyde and methylisonicotinate in concentration 10^{-2} M and does not form the corresponding NAD-analogues in the exchange reaction. Since NADase from the lizard liver mitochondria shows similar exclusively hydrolytic properties, the double function of these enzymes postulated by Kaplan is discussed.

2. Formation of the histamine derivative of APPR from NAD in the presence of carp NADase was found to result from the non-enzymic reaction of histamine (probably its amino-N) with APPR formed by the enzyme.

REFERENCES

- [1] Abdel-Latif A. A. & Alivisatos S. G. A. - *J. Biol. Chem.* **237**, 500, 1962.
- [2] Alivisatos S. G. A. - *Nature* **183**, 1034, 1959.
- [3] Alivisatos S. G. A. & LaMantia L. - *Biochem. Biophys. Res. Comm.* **2**, 164, 1960.
- [4] Alivisatos S. G. A., LaMantia L. & Matijevitch B. L. - *Biochim. Biophys. Acta* **58**, 201, 1962.

- [5] Alivisatos S. G. A., LaMantia L. & Matijevitch B. L. - *Biochim. Biophys. Acta* **58**, 209, 1962.
- [6] Alivisatos S. G. A., Ungar F., Lucacs L. & LaMantia L. - *J. Biol. Chem.* **235**, 1742, 1960.
- [7] Colowick S. P., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 1, p. 98. Acad. Press, New York 1960.
- [8] Hanes C. S. & Isherwood F. A. - *Nature* **164**, 1107, 1949.
- [9] Horecker B. L., Smyrniotis P. Z. & Klenow H. - *J. Biol. Chem.* **205**, 661, 1953.
- [10] Kaplan N. O., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 2, p. 660. Acad. Press, New York 1960.
- [11] Kaplan N. O. & Ciotti M. M. - *J. Biol. Chem.* **221**, 823, 1956.
- [12] Kaplan N. O., Ciotti M. M., von Eys J. & Burton R. M. - *J. Biol. Chem.* **234**, 134, 1959.
- [13] Kaplan N. O., Ciotti M. M. & Stolzenbach F. E. - *J. Biol. Chem.* **221**, 833, 1956.
- [14] Kaplan N. O., Colowick S. P. & Nason A., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 904. Acad. Press, New York 1960.
- [15] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **2**, 279, 1955.
- [16] Raczyńska-Bojanowska K. & Gąsiorowska I. - *Acta Biochim. Polon.* **10**, 117, 1963.
- [17] Zatman L. J., Kaplan N. O. & Colowick S. P. - *J. Biol. Chem.* **200**, 197, 1953.
- [18] Zatman L. J., Kaplan N. O., Colowick S. P. & Ciotti M. M. - *J. Biol. Chem.* **209**, 453, 1954.
- [19] Zatman L. J., Kaplan N. O., Colowick S. P. & Ciotti M. M. - *J. Biol. Chem.* **209**, 467, 1954.

BADANIA NAD REAKCJĄ IMIDAZOLIZY I WYMIANY PRZY UŻYCIU NADazy Z MITOCHONDRII WĄTROBY KARPIA

Streszczenie

Wykazano, że około 100-krotnie oczyszczona NADaza z mitochondrii wątroby karpia nie jest hamowana przez INH, etylnikotynian, 3-acetylopirydynę i 3-aldehydopirydynę w stężeniu 10^{-2} M, jak również nie tworzy analogów NAD w reakcji wymiany, w której amid kwasu nikotynowego jest zastąpiony przez pochodne pirydynowe. Podobne własności wykazuje NADaza otrzymana z mitochondrii wątroby jaszczurki, w związku z czym poddano dyskusji hipotezę Kaplana dotyczącą podwójnej funkcji tych enzymów.

Powstawanie histaminowej pochodnej APPR z NAD w obecności NADazy z mitochondrii wątroby karpia jest wynikiem nieenzymatycznej reakcji histaminy (prawdopodobnie jej grupy aminowej) z APPR wytworzonym enzymatycznie z NAD.

Received 4 January 1964.

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THE COMPLEXES OF NADH AND NAD WITH LIVER ALCOHOL DEHYDROGENASE AT pH 10*The Nobel Medical Institute, Department of Biochemistry, Stockholm, Sweden*

Whereas the dissociation constants of binary complexes of liver alcohol dehydrogenase (LADH) with NADH and NAD^+ ($K_{E,R}$ and $K_{E,O}$) in the pH region 6 to 9 were previously determined [7] by using spectrophotofluorometric methods, these values at higher pH were still unknown.

Theorell & Bonnicksen [5] and Theorell & Chance [6] in 1951 concluded from spectrophotometrical titrations of LADH with increments of NADH that each molecule of LADH binds 2 molecules of NADH at pH 6 to 9. At pH 10 the data obtained seemed to indicate a ratio of one to one. The absorption differences caused by the maximum shift from 340 to 325 m μ when NADH was coupled to LADH were used as indication.

However, the data obtained at that time were not sufficiently accurate to prove whether one NADH was bound comparatively tightly, or two NADH much more loosely. Later it was found by McKee & Donninger [4] that one LADH at pH 10 in the presence of the ternary ligand, isobutyramide [14, 8, 9], binds 2 molecules of NADH, and Theorell & Yonetani [12] proved that the ratio LADH:NAD:pyrazole in ternary complex was 1:2:2 at pH 10.

It therefore seemed interesting to determine the dissociation constants $K_{E,R}$ and $K_{E,O}$ at pH 10 using higher concentration of enzyme and coenzymes than previously, under the assumption that rather highly dissociated binary compounds were formed. Under such conditions fluorometry is not well applicable because of quenching by light absorption. On the other hand spectrophotometry at these higher concentrations is much more suitable.

$K_{E,R}$ was determined directly by titrating LADH with increments of NADH. $K_{E,O}$ was determined from the apparent $K_{E,R}$ in the presence of NAD [7]. The wavelengths used for the light absorption measurements were 355 m μ , where the extinction coefficient shows its maximal change, $\Delta\epsilon = 2.5 \text{ mM}^{-1} \times \text{cm}^{-1}$ [5, 11] and 329 m μ which is the isosbestic point for free and bound NADH, $\epsilon = 5.8 \text{ mM}^{-1} \times \text{cm}^{-1}$.

Double difference spectrophotometry was used [13].

Materials

LADH was prepared from horse liver and recrystallized four times in 10% ethanol according to a modification of Dalziel's method [1]. The enzyme preparation, which was stored at -20° in 30% ethanol, was collected by centrifugation. The sedimented crystals were dissolved in $\text{NH}_3\text{-HCl}$ buffer, pH 9.6, ionic strength 0.1 and twice recrystallized in 0.001 M-sodium phosphate buffer, pH 7, containing 10% methanol. The methanol-crystallized enzyme was then dialysed against 0.01 M-sodium phosphate buffer, pH 7, for several days with frequent changes of outer media. The concentration of the dialysed enzyme preparation was determined spectrophotometrically by titrating LADH with NAD^+ in the presence of excess pyrazole [12] and was expressed as N , the normality of coenzyme-binding capacities per liter. The purity of the enzyme preparation was found to be 100% on the basis of a specific extinction coefficient at 280 $m\mu$ of 0.42 ml./mg. \times cm. and a molecular weight of 84 000 per two coenzyme-binding sites [2]. NADH (95% purity) and NAD^+ (98% purity) were purchased from Sigma Chemical Co. The coenzyme concentration was expressed on the basis of a difference-extinction coefficient at 340 $m\mu$ (reduced minus oxidized) of $6.22 \text{ mm}^{-1} \times \text{cm.}^{-1}$ [3].

Methods

The interaction of LADH with coenzymes in the presence and absence of substrate-competitive inhibitors such as caprate and isobutyramide,

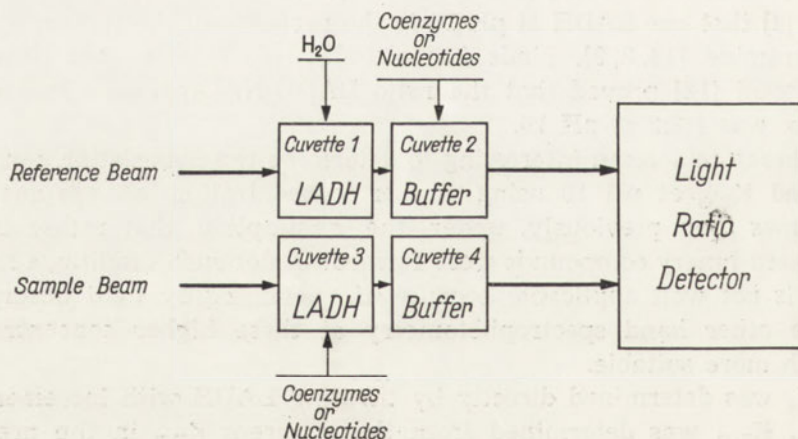
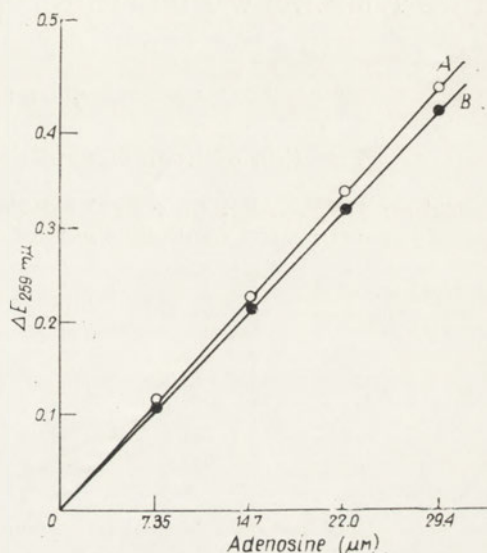


Fig. 1. Arrangement of cuvettes for double-difference spectrophotometry.

was studied with a Beckman DK-2 ratio recording spectrophotometer. In order to observe relatively small light absorption changes in strongly light absorbing backgrounds, double difference spectrophotometry [13]

was employed, in which two pairs of well matched quartz-cuvettes were simultaneously used (cf. Fig. 1). Cuvettes 1 and 3 were filled with 1.5 ml. of between 30 and 100 μN solutions of LADH, whereas cuvettes 2 and 4 were filled with 1.5 ml. of appropriate buffers. A difference spectrum between these pairs (3 + 4 minus 1 + 2) was a straight line which was used as a base line of the measurements. 5 μl .-aliquots of coenzyme or nucleotide solutions were added to cuvettes 2 and 3 and the same volume of distilled water was added to cuvette 1. Then the difference spectrum

Fig. 2. Demonstration of the linear absorbancy response of the instrument in ordinary (A) and double-difference (B) spectrophotometries. Titrations were carried out upon adding 5 μl .-aliquots of 2.20 mM-adenosine to 1.5 ml. of reaction mixtures. (A), Sample and reference cuvettes contained 1.5 ml. of sodium phosphate buffer, pH 7.0, ionic strength 0.1. (B), Sample and reference cuvettes contained 1.5 ml. of the same buffer containing 50 μN -LADH and 130 μM -adenosine. Thus the total light-absorptions at 259 $\text{m}\mu$ of the reaction mixture before the titrations were approximate absorbancies of zero and 3.0 for (A) and (B), respectively.



was recorded from 250 to 400 $\text{m}\mu$. When the total absorption in the reference beam exceeded an absorbancy of 2.0, the mechanical response of the instrument became too slow to follow the absorption changes by the automatic wavelength scanning, so that the spectra were recorded manually point by point. If this precaution was taken, the instrument responded linearly to absorption changes even when the total absorption in the reference beam exceeded an absorbancy of 3.0 (cf. Fig. 2).

Millimolar difference-extinction coefficients ($\Delta\epsilon_{\text{mM}}$) of dissociable binary or ternary complexes were calculated. On the basis of these $\Delta\epsilon_{\text{mM}}$ values the dissociation constants of LADH-complexes were directly calculated from the absorption changes occurring at varied concentrations of the reactants. The spectrophotometric method for the determination of the dissociation for LADH-NAD-caprate complexes is more straightforward than the fluorometric ones [10, 14, 7, 9] in which the competition between NADH and NAD^+ was used.

RESULTS

Table 1 gives the average experimental data from 3 titrations carried out under the conditions given in the Table. The average result was $K_{E,R} = 10 \mu\text{M}$. Tables 2 and 3 summarize the results from two titrations of LADH + NAD^+ with increments of NADH. When comparatively high concentrations of LADH and NAD^+ are mixed a certain reduction of NAD^+ to NADH is inevitable. This NADH was determined from its absorption at $329 \text{ m}\mu$, and the results given as ΔR in the second columns of the Tables. ΔR was subtracted from the total concentration of NAD.

Table 1

Titration of liver alcohol dehydrogenase with NADH

Conditions: $32.5 \mu\text{N}$ -LADH (E) + $6 \times 13.7 \mu\text{M}$ -NADH (R); pH 10; 0.1 M-glycine - NaOH; 23.5° . $\epsilon_{329} = 5.8 \text{ mm}^{-1} \times \text{cm}^{-1}$ (isosbestic wavelength for R and ER); $\Delta\epsilon_{355} = 2.5 \text{ mm}^{-1} \times \text{cm}^{-1}$

Addition of R no.	[R _{total}]	[R _{free}]	[ER]	[E _{free}]	[K _{ER}]
1	13.7	4.6	9.1	23.4	12
2	27.4	10.7	16.7	15.8	10
3	41.1	19.5	21.6	10.9	10
4	54.8	29.8	24.6	7.9	10
5	68.5	41.7	26.8	5.7	9
6	82.2	53.8	28.4	4.1	8
Average $10 \mu\text{M}$					

Table 2

Titration of LADH-NAD with NADH

Conditions: $32.5 \mu\text{N}$ -LADH (E) + $56.8 \mu\text{M}$ -NAD (O) titrated with $5 \times 13.7 \mu\text{M}$ -NADH (R); pH 10; 0.1 M-glycine - NaOH; 23.5° ; $\Delta\epsilon_{355} = 2.5 \text{ mm}^{-1} \times \text{cm}^{-1}$.

Addition of R no.	ΔR (μM)	[R _{total}]	[R _{free}]	[ER]	[O _{total}]	[O _{free}]	[EO]	[E _{free}]	[K _{E,O}]
1	5.1	18.8	12.7	6.1	51.7	30.1	21.6	4.8	7
2	4.7	32.1	21.3	10.8	52.1	35.5	16.6	5.1	11
3	4.7	45.8	31.5	14.3	52.1	38.5	13.6	4.6	13
4	4.7	59.5	43.6	15.9	52.1	39.1	13.0	3.6	11
5	4.7	73.2	56.2	17.0	52.1	36.6	12.5	3.0	9
Average $10 \mu\text{M}$									

Table 3

Titration of LADH-NAD with NADH

Conditions: 32.5 μM -LADH (E) + 142 μM -NAD (O) titrated with $4 \times 13.7 \mu\text{M}$ -NADH (R); pH 10; 0.1 M-glycine - NaOH; 23.50; $\Delta\epsilon_{355} = 2.5 \text{ mm}^{-1} \times \text{cm}^{-1}$.

Addition of R no.	ΔR (μM)	$[R_{\text{total}}]$	$[R_{\text{free}}]$	$[ER]$	$[O_{\text{total}}]$	$[O_{\text{free}}]$	$[EO]$	$[E_{\text{free}}]$	$[K_{E,O}]$
1	9.3	23.0	18.6	4.4	133	107	25.7	2.36	10
2	9.2	36.6	29.9	6.7	133	109	23.6	2.24	10
3	9.4	50.5	42.4	8.1	133	110	22.5	1.88	9
4	9.7	64.5	54.5	10.0	132	111	21.7	1.84	10
Average 10 μM									

The spectrophotometry then as before gave the concentrations of free (R) and bound (ER) NADH. An unknown amount of the free LADH was bound to NAD at "EO". [EO] was calculated by using the value

$$K_{E,R} = 10 \mu\text{M} = \frac{(E_{\text{total}} - ER - EO)(R_{\text{total}} - ER)}{ER}$$

where only EO is unknown.

$$K_{E,O} = \frac{(E_{\text{total}} - ER - EO)(O_{\text{total}} - EO)}{EO}$$

DISCUSSION

$K_{E,R}$ and $K_{E,O}$ at pH 10 were both found to be 10 μM . $K_{E,R}$ in the pH region 6 to 9 varies parallelly with $K_{E,A}$ (A, adenosine diphosphate ribose), $K_{E,A}$ being 80 - 100 times larger than $K_{E,R}$ [13, 15]. At pH 10, $K_{E,A}$ is rather uncertain and may in fact be as high as 800 μM . On the whole, the present experiments at pH 10 verify our earlier findings [13, 15] that the variations of $K_{E,R}$ with pH entirely depend upon the "specific" binding site for the adenosine diphosphate ribose (ADPR) moiety. The dihydropyridine moiety must be close to, and possibly bound to the active Zn atoms. At this "active" site the firmness of the binding is increased by a factor of 80 - 100, independent of pH variations from 6 to 10. The chemical nature of this bond remains uncertain.

The value of $K_{E,O}$ found at pH 10 (10 μM) is probably somewhat high, because at pH 9 Theorell & McKee [8] observed that 0.1 M-glycine buffer had a slightly dissociating effect on EO, caused by complex formation of glycine with the Zn. In the absence of glycine we would expect $K_{E,O}$ at pH 10 to be 7 - 8 μM in agreement with: first, the value for $K_{E,O}$ pH 10, calculated by Theorell & McKee [8] ($K_{E,O} = 8.5 \mu\text{M}$) assuming

that the values determined at pH 6 to 9 fitted to a monovalent dissociation curve with pK 7.07; secondly, with the dissociation constant $K_{E,O}$ $7.6 \mu M$ [8] where I is caprate. This $K_{E,O}$ is independent of pH.

As discussed previously [9] these results strongly suggest that a repulsion between Zn^{2+} and pyridinium is the reason for the high values of $K_{E,O}$ at low pH. This repulsion can be cancelled either by increasing pH until a water molecule at the Zn^{2+} loses a proton and leaves a hydroxyl group; or by substituting this water molecule by a group which stays negatively charged in the whole pH region 6 to 10, for example caprate. The results presented in this paper give strong support to this theory by demonstrating that the experimentally determined value of $K_{E,O}$ at pH 10 agrees with the value which was formerly calculated on the assumptions mentioned above.

One of the questions still needing to be answered is the following. When pH is raised from 9 to 10, both $K_{E,R}$ and $K_{E,A}$ increase at least tenfold, whereas $K_{E,O}$ goes down a little and $K_{E,O}$ (I, caprate) stays constant. This is unexpected if the ADPR moiety in NAD^+ , $NADH$ and free ADPR would have the same conformation. It is perhaps possible that the oxidation of $NADH$ to NAD^+ is connected with some change in the ADPR part.

The author is indebted for financial supports given by Statens Medicinska Forskningsråd, Statens Naturvetenskapliga Forskningsråd, Knut och Alice Wallenbergs Stiftelse, The Rockefeller Foundation, and Svenska Maltdrycksforskningsinstitutet.

SUMMARY

The dissociation constants of the binary complexes of liver alcohol dehydrogenase with $NADH$ ($K_{E,R}$) and with NAD^+ ($K_{E,O}$) at pH 10 were determined by the aid of double difference spectrophotometry at 329 and 355 $m\mu$. They were both found to be 10 μM . This gives strong support to earlier theories on the mode of binding between the coenzymes and liver alcohol dehydrogenase.

REFERENCES

- [1] Dalziel K. - *Acta Chem. Scand.* **12**, 459, 1958.
- [2] Ehrenberg A. & Dalziel K. - *Acta Chem. Scand.* **12**, 465, 1958.
- [3] Horecker B. L. & Komberg A. - *J. Biol. Chem.* **175**, 385, 1948.
- [4] McKee J. S. M. & Donninger C. - *Biochem. J.* **85**, 23P, 1962.
- [5] Theorell H. & Bonnichsen R. - *Acta Chem. Scand.* **5**, 1105, 1951.
- [6] Theorell H. & Chance B. - *Acta Chem. Scand.* **5**, 1127, 1951.
- [7] Theorell H. & McKee J. S. M. - *Acta Chem. Scand.* **15**, 1797, 1961.
- [8] Theorell H. & McKee J. S. M. - *Acta Chem. Scand.* **15**, 1811, 1961.

- [9] Theorell H. & McKee J. S. M. - *Acta Chem. Scand.* **15**, 1834, 1961.
- [10] Theorell H. & Winer A. D. - *Arch. Biochem. Biophys.* **83**, 291, 1959.
- [11] Theorell H. & Yonetani T. - *Arch. Biochem. Biophys.* Suppl. 1, 209, 1962.
- [12] Theorell H. & Yonetani T. - *Biochem. Z.* **338**, 537, 1963.
- [13] Yonetani T. - *Biochem. Z.* **338**, 300, 1963.
- [14] Winer A. D. & Theorell H. - *Acta Chem. Scand.* **14**, 1729, 1960.
- [15] Yonetani T. - *Acta Chem. Scand.* **17**, Suppl. 1, 96, 1963.

KOMPLEKSY NADH I NAD Z DEHYDROGENAZĄ ALKOHOLOWĄ Z WĄTROBY PRZY pH 10

Streszczenie

Przy zastosowaniu różnicowej spektrofotometrii przy dwóch długościach fali 329 i 355 m μ oznaczono stałe dysocjacji kompleksów wątrobowej dehydrogenazy alkoholowej z NADH($K_{E,R}$) i z NAD⁺($K_{E,O}$). Obie wartości wynosiły 10 μ M. Wyniki te wskazują na słuszność wcześniejszych poglądów dotyczących rodzaju wiązania między koenzymami a wątrobową dehydrogenazą alkoholową.

Received 11 January 1964.

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**FRACTIONATION OF HISTONES FROM CELL NUCLEI
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Previous work from this Laboratory on ox pancreas deoxyribonucleoproteins (DN-proteins) was mainly concerned with histones isolated from DN-protein obtained from whole tissue homogenate [2, 6, 7, 8]. The chromatographic analysis of these proteins on carboxymethylcellulose according to Phillips & Johns [11] has shown two fractions moderately rich in lysine and with the same ratio of lysine to arginine (2.0). Fractional preparation according to Daly & Mirsky [1] gave two fractions: one arginine-rich and another moderately lysine-rich [8]. These results were attributed to a contamination of histones with ribonucleoproteins. It appeared therefore necessary to fractionate histones obtained from the DN-protein isolated from highly purified and gelling nuclei. The extraction of histones from the material free from cytoplasmic and nuclear ribonucleoprotein seemed to assure their purity.

Pancreatic cell nuclei, microscopically pure, gelling and in good yield, were isolated, by applying the technique of Neelin & Butler [9], from ox pancreas obtained from the slaughter-house and put in ice 10 min. after the death of the animal. The chromatography of histones was carried out on a carboxymethylcellulose (CM-cellulose) column according to Phillips & Johns [11]. CM-cellulose was obtained by the method of Peterson & Sober [10]. The slightly modified [8] technique of Daly & Mirsky [1] was applied for fractional precipitation.

The histones were hydrolysed with 6 N-HCl at 110° for 18 hr. and the amino acid composition was established by the method of Kalinkievič [5] and according to Durrum's technique [4] adapted for the quantitative determination of amino acid composition [7].

Chromatography of histones

Four histone preparations isolated from pure nuclei were fractionated on a CM-cellulose column. An example is given in Fig. 1. Two main fractions, F_2 and F_3 , were eluted with 0.01 and 0.02 N-HCl, and two smaller ones with 0.002 and 0.04 N-HCl.

The amount of nitrogen was determined in the separate fractions and, as may be seen from the results presented in Table 1, 79–90% of the histone nitrogen was recovered. Fractions F_2 and F_3 contained together

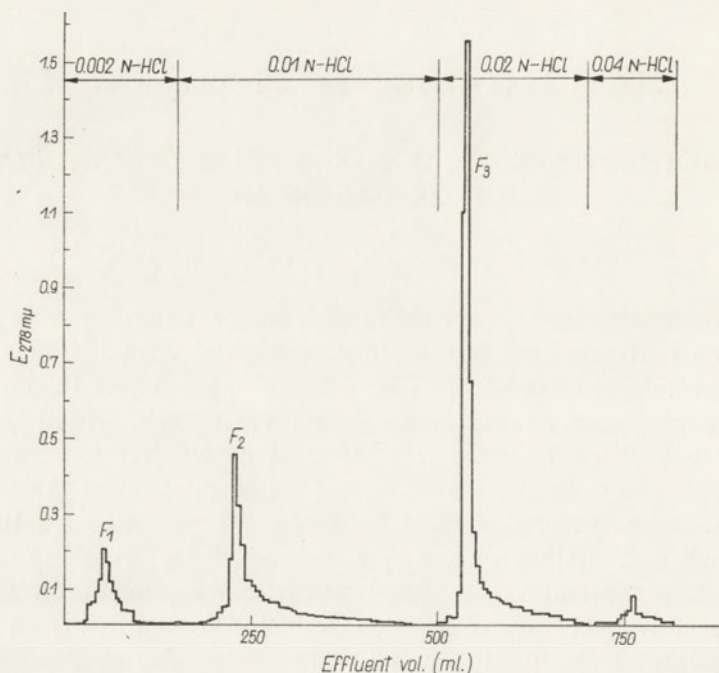


Fig. 1. Elution diagram of histone preparation *J* from ox pancreas nuclei chromatographed on a carboxymethylcellulose column (14 × 2 cm.).

about 94% of the eluted nitrogen and about 81% of the nitrogen of the fractionated histone. The corresponding values for these fractions taken separately were for F_2 42% and 36%, and for F_3 52% and 45%.

Fractions F_2 and F_3 were hydrolysed and the content of amino acids

Table 1

Fractionation of histones on a carboxymethylcellulose column

In the separated fractions the total nitrogen was determined and expressed as mg. N per 100 mg. of the starting histone.

Preparation	Nitrogen in starting histone (%)	Fraction eluted with HCl (N)						Recovery
		0.002 F_1	0.01 F_2	0.02 F_3	0.04	0.1	0.2	
<i>C</i>	16.2	0.21	6.10	7.23	0.49	0.12	0.07	14.22
<i>J</i>	17.8	0.21	5.94	7.18	0.59	0.08	0.04	14.04
<i>N</i>	16.9	0.28	6.00	8.12	0.62	0.10	0.05	15.17
<i>XXV</i>	16.2	0.28	6.12	7.54	0.44	0.08	0.04	14.50

was determined (Table 2). Both fractions appeared to be rich in basic amino acids; the nitrogen of arginine, lysine and histidine amounted to 39% and 42%, resp., of total nitrogen. Acidic amino acids were present in smaller amounts (8 and 8.6% of total N, resp.). The nitrogen of neutral amino acids in both fractions accounted for 37% of total nitrogen. The

Table 2

Amino acid composition of fractions F_2 and F_3 from the CM-cellulose column

The amino acid content is expressed as: (a), grams of amino acid per 100 g. of protein; (b), amino acid N as percentage of total nitrogen; (c) moles of amino acid per 100 moles of all amino acids found. Mean values for 4 histone preparations are given.

Amino acid	Fraction F_2			Fraction F_3		
	a	b	c	a	b	c
Arginine	8.4	17.5	7.4	9.8	19.0	7.8
Lysine	14.2	17.6	15.0	16.4	18.9	15.6
Histidine	2.0	3.6	2.0	2.3	4.0	2.1
Aspartic acid	4.5	3.1	5.2	5.3	3.4	5.6
Glutamic acid	7.8	4.8	8.1	9.0	5.2	8.6
Serine	3.7	3.2	5.3	4.1	3.3	5.4
Glycine	5.7	6.9	11.6	3.9	4.4	7.2
Threonine	4.3	3.3	5.5	4.8	3.4	4.2
Alanine	6.5	6.6	11.2	8.1	7.6	12.7
Tyrosine	3.8	1.9	3.2	2.3	1.1	1.8
Valine	4.4	3.4	5.8	5.4	3.9	6.4
Leucine-iso-leucine	10.5	7.2	12.3	12.3	7.8	13.1
Phenylalanine	1.9	1.9	1.8	2.4	1.2	2.0
Proline	3.9	3.1	5.2	5.9	4.3	7.1
Tryptophan	0.14	0.13	0.10	0.18	0.16	0.12
Sum	81.74	84.23		92.18	87.66	

amino acid composition of fractions F_2 and F_3 was fairly similar. A somewhat higher content of all amino acids was found in F_3 except for glycine and tyrosine which prevail in F_2 . Tryptophan was found in both fractions (0.14% in F_2 and 0.18% in F_3).

Fractional precipitation of histones

Histone preparations obtained from the DN-protein extracted from pancreas nuclei were submitted to isoelectric precipitation (Table 3); from 100 mg. of the whole histone preparation, 35 - 44 mg. was precipitated at pH 10.6 (fraction I) and 10 - 13 mg. at pH 6.0 (fraction II).

Table 3

Fractional precipitation of histones from ox pancreas nuclei

Histones (100 mg.) isolated from deoxyribonucleoprotein were submitted to isoelectric precipitation at pH 10.6 and 6.0.

Preparation	Fraction I, pH 10.6 (mg.)	Fraction II, pH 6.0 (mg.)
<i>K</i>	36.7	12.1
<i>S</i>	43.5	11.4
<i>Z</i>	38.2	10.5
<i>XIV</i>	44.8	12.7
<i>XX</i>	35.3	10.6

By the isoelectric precipitation applied, only 46 - 58% of the histone was recovered. The loss in the material is probably due to the purification procedure (repeated dissolution, precipitation, centrifugation and dialysis) and also to the low temperature applied.

Table 4

Amino acid composition of fractions I and II obtained by isoelectric precipitation

The amino acid content is expressed as: (a), grams of amino acid per 100 g. of protein; (b), amino acid N as percentage of total nitrogen; (c) moles of amino acid per 100 moles of all amino acids found. Mean values are given for 5 preparations described in Table 3.

Amino acid	Fraction I			Fraction II		
	a	b	c	a	b	c
Arginine	14.2	26.8	11.0	7.0	14.9	5.7
Lysine	12.1	13.5	11.1	18.4	23.0	17.8
Histidine	2.4	3.9	2.1	1.5	2.7	1.4
Aspartic acid	6.0	3.7	6.1	3.9	2.7	4.2
Glutamic acid	10.5	5.9	9.6	8.0	5.0	7.7
Serine	3.8	2.8	4.8	4.2	3.6	5.6
Glycine	4.2	4.6	7.6	3.7	4.6	7.1
Threonine	5.3	3.8	6.0	4.2	3.2	5.0
Alanine	7.2	6.7	11.0	11.0	11.4	17.5
Tyrosine	3.8	1.7	2.8	2.1	1.1	1.6
Valine	7.4	5.2	8.4	5.6	4.4	6.9
Leucine-iso-leucine	12.6	7.8	12.9	8.1	5.7	8.8
Phenylalanine	3.0	1.4	2.4	3.2	1.8	2.7
Proline	3.3	2.4	3.8	6.3	5.0	7.7
Tryptophan	0.19	0.15	0.12	0.10	0.09	0.07
Sum	95.99	90.35		87.30	89.19	

The mean content of amino acids in the precipitated fractions is given in Table 4. The nitrogen of basic amino acids represented about 44% of total nitrogen in fraction I and 41% in fraction II. Fraction I was much richer in nitrogen of arginine than that of lysine while in fraction II these relations were inverted. In fraction I the nitrogen of acidic amino acids represented 9.6%, and that of neutral amino acids 36.6% of its total nitrogen; the corresponding data for fraction II were 7.7 and 41%.

DISCUSSION

In the previous work [8] the chromatography of histones and their amino acid composition were studied on whole homogenates of ox pancreas; the results obtained presently with histones isolated from purified nuclei proved to be similar but they differ considerably from the results of separation of calf thymus histone reported by Phillips & Johns [11]. In thymus histone preparations, the main fraction was the F_2 eluted by 0.01 N-HCl and the $F_2:F_3$ ratio was 2.2 - 2.5 while for the pancreas histone it was 0.7 - 0.8.

The amino acid composition of the two column fractions of ox pancreas nuclei was similar. They both contained more lysine than arginine but F_2 contained more glycine and tyrosine and less proline and glutamic acid than F_3 , closely resembling the F_2 and F_3 fractions of histones from the whole pancreas homogenate [8]. They differed, however, from the corresponding histones of calf thymus [11]. Both fractions of the pancreas histone had a similar ratio of basic to acidic amino acids (about 1.8) whereas F_2 of the thymus histone had a higher ratio (1.96). The ratio of lysine to arginine in both fractions of pancreas histone was about 2 while F_3 of the thymus histone had a lower value (1.54). Fractionation on a CM-cellulose column by the method of Phillips & Johns [11] of thymus and pancreas histones gives two fractions moderately rich in lysine, whereas the content of arginine is smaller in F_2 than in F_3 of the thymus histone.

The isoelectric fractionation of histones from pancreas nuclei by the method of Daly & Mirsky [1] gives, similarly as with histones from the whole homogenate, two fractions. The differences in amino acid composition between these two fractions are larger than those between fractions obtained by chromatography on a CM-cellulose column. Fraction II precipitated at pH 6.0 is richer in lysine than in arginine whereas fraction I precipitated at pH 10.6 contains almost the same quantities of arginine and lysine. Apart from that, fraction I contains considerably more arginine, histidine, acidic amino acids, valine, leucine with isoleucine and tryptophan, but less alanine and lysine, than fraction II. The comparison of fractions I and II obtained from ox pancreas and from calf thymus histones [1] shows a great similarity of fractions

obtained at pH 10.6. Their lysine:arginine ratio is 1.0 and the ratio of basic to acidic amino acids is 1.5 for the pancreas histone and 1.6 for the thymus histone. The molar amounts of the remaining amino acids are similar except that in the pancreas the content of valine and leucine with isoleucine is higher, and that of glycine and alanine lower than in thymus histones. The content of tryptophan in the pancreas histone is 0.12 mole. Fraction II of the thymus histone contains no histidine. The molar ratio of lysine to arginine is 11 for this fraction; for the pancreas histone, however, it is only 3. The ratio of basic to acidic amino acids for fraction II of thymus is 6. The thymus histone precipitated at pH 6.0 is very lysine-rich; the corresponding fraction II of pancreas histone is only moderately lysine-rich. This difference might be due to a slight difference in the processes applied for the precipitation of both types of histones (at room temperature for thymus, at 0° and below for pancreas). Presumably the fraction very lysine-rich cannot be entirely precipitated at a low temperature and its presence in the precipitate obtained at pH 6.0 affects the amino acid composition of fraction II of the pancreas histone. The effect of prolonged dialysis on fraction II of ox pancreas histone should also be envisaged.

SUMMARY

Histones isolated from pure nuclei of ox pancreas were fractionated on a carboxymethylcellulose column and by isoelectric precipitation. The amino acid composition of the fractions obtained was similar to that of histones from the whole tissue homogenate. The fractions obtained were compared with the corresponding fractions from calf thymus histone.

REFERENCES

- [1] Daly M. M. & Mirsky A. E. - *J. Gen. Physiol.* **38**, 405, 1955.
- [2] Dmochowski A., Khouvine Y. & Kłyszajko L. - *Zeszyty Naukowe UŁ*, seria II, **11**, 1961.
- [3] Dounce A. L. & Mounthy K. J. - *J. Biochem. Biophys. Cytol.* **1**, 155, 1955.
- [4] Durrum E. L. - *J. Am. Chem. Soc.* **72**, 2943, 1950.
- [5] Kalinkievič A. F. & Udovenko G. V. - *Dokl. Akad. Nauk S.S.S.R.* **126**, 684, 1959.
- [6] Kłyszajko L. & Khouvine Y. - *Bull. Soc. Chim. Biol.* **42**, 761, 1960.
- [7] Kłyszajko L. & Khouvine Y., *Dezoksyrybonukleoproteidy trzustki wołowej. Cz. I. Badanie składu chemicznego*. Łódzkie Towarzystwo Naukowe, Nr. 88, Łódź 1962.
- [8] Kłyszajko L. & Khouvine Y. - *Bull. Soc. Chim. Biol.* **46**, 111, 1964.
- [9] Neelin J. M. & Butler G. E. - *Canad. J. Biochem. Physiol.* **37**, 843, 1959.
- [10] Peterson E. A. & Sober H. A. - *J. Am. Chem. Soc.* **78**, 751, 1956.
- [11] Phillips D. M. P. & Johns E. W. - *Biochem. J.* **72**, 538, 1959.

FRAKCJONOWANIE HISTONÓW Z JĄDER KOMÓRKOWYCH
TRZUSTKI WOŁOWEJ

Streszczenie

Histony z czystych jąder komórkowych trzustki wołowej rozfrakcjonowano na karboksymetylocelulozie oraz przez strącanie izoelektryczne. Zanalizowano skład aminokwasowy otrzymanych frakcji i stwierdzono ich duże podobieństwo do odpowiednich białek otrzymanych z dezoksynukleoproteidów izolowanych z pełnego homogenatu tkankowego. Przedyskutowano różnice między otrzymanymi frakcjami a odpowiednimi frakcjami z grasicy cielęcej.

Received 11 January 1964.

M. SCHMIDT and A. DMOCHOWSKI

THE ACID MUCOPOLYSACCHARIDES OF NORMAL AND ATHEROSCLEROTIC HUMAN AORTA

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In view of the frequent association of acid mucopolysaccharides and atheromatic deposits within the human aorta wall, the suggestion has been made that these acid mucopolysaccharides (glycosaminoglycans) present in the connective tissue may be in some way associated with the primary atherogenic process. It was assumed that their deposition constitutes the initial stage in the development of atherosclerotic lesions in the human artery wall. Histochemical investigations of acid mucopolysaccharides (acid MPS) in human aorta wall show, owing to their metachromatic properties, that under normal conditions the metachromatic material is present only in the inner third part [1, 12]. The intensity of the metachromasia, however, increases progressively with age [12]. From morphological studies it is obvious that acid MPS accumulate within limited focal regions; at the same time deposition of lipid and calcium takes place [1, 10, 21, 22]. Significant changes in the pattern of acid MPS of skin, cartilage and aorta concomitant with age, have been described in recent years. Of particular interest are the changes concerning the aorta wall. Dyrbye & Kirk [9] extracted a mixture of acid MPS from human aorta and showed that the main component was a sulphated polysaccharide, with an additional smaller amount of a non-sulphated compound. This sulphated polysaccharide was depolymerized by testicular hyaluronidase; bacterial hyaluronidase, on the contrary, was active only on the non-sulphated polysaccharide [10]. It has been suggested that this sulphated polysaccharide is a form of chondroitin sulphates, which accumulates in regions of intimal and medial degeneration and interacts in some way with the plasma lipoproteins and fibrinogen to produce focal lipid- and fibrin-deposits [21, 22]. No change in the galactosamine to glucosamine ratio was noticed by these authors until the age of 60. In older age they observed only a slight increase. Bertelsen [3] demonstrated an increase of hexosamine with age. Similar results were reported by Buddecke [7] who established

also an increase of both acid MPS and neutral mucopolysaccharides (mucoids) in human atherosclerotic aorta. While most of these studies were based on the analysis of the whole aorta for hexosamine, uronic acid or sulphate, only few works have been devoted to the identification and separation of the individual acid MPS of the aorta wall. Kaplan & Meyer [14] analysed 43 human aortae, classified into 7 separate groups of different age. These authors found no difference in the total amount of acid MPS in relation to age. The individual acid MPS were identified as: hyaluronic acid, chondroitin-6-sulphate (type C), dermatan sulphate (chondroitin sulphate B), and heparan sulphate (heparitin sulphate). Heparin, chondroitin-4-sulphate (type A) or keratan sulphate were always absent. Kaplan & Meyer demonstrated also dramatic changes in the behaviour of the individual acid MPS of human aorta during the aging process. In general, dermatan sulphate and heparan sulphate increase significantly with age, while hyaluronic acid and chondroitin-6-sulphate decrease markedly. In disagreement with these results, Buddecke [7] found a mixture of acid MPS composed of keratan sulphate, chondroitin, hyaluronic acid, chondroitin-4-sulphate, and dermatan sulphate. He reported further, like Kaplan & Meyer [14] a decrease of hyaluronic acid and significant increase of dermatan sulphate. Bertelsen & Jensen [4] isolated both hyaluronic acid and chondroitin sulphates from aged human aortae. They assumed that the increase in the ratio of chondroitin sulphate to hyaluronic acid is concomitant with age. More recently, Bertelsen & Marcker [5] reported that the increase of total acid MPS in aged human aorta is due to that of sulphated acid MPS (undigestable fraction) which they called chondroitin sulphates.

The present paper describes some experiments undertaken to compare the total amount of acid MPS in each single aorta, both normal and atherosclerotic, and to investigate the pattern of the individual acid MPS in both cases.

MATERIAL AND METHODS

Isolation of acid mucopolysaccharides. Ninety human aortae were taken at autopsy 10-12 hr. after death. After removal of the adherent tissue, the aortae were stored in acetone until a sufficient number of specimens were collected. The adventitia was then removed carefully by hand and the degree of atherosclerotic alterations was examined according to Bertelsen & Marcker [5]. By means of their classification the whole material was divided into the following groups: I, newborn; II, normal, no medial calcium; III, normal, medial calcium (physiosclerotic); IV, slight atherosclerotic alterations; V, distinct atherosclerotic alterations; VI, profound atherosclerotic alterations.

None of the patients had suffered from rheumatic fever, rheumatoid arthritis or any other collagen disease. None had been treated with corticosteroids. Each acetone-dried aorta was disintegrated, delipidated with a hot mixture of ethanol-acetone (1:1) and ether. The delipidated aorta was suspended in 2 M-versenate (10 ml. per g. tissue) and shaken overnight. After this time the tissue showed no appreciable amounts of calcium. The tissue was dehydrated with acetone, air-dried and weighed. It was then digested with pure papain (CalBiochem., St. Nicolausen, Switzerland) as described by Buddecke [7].

The acid MPS were precipitated as calcium salts and deproteinized according to Meyer *et al.* [16]. The nitrogen content of the mixture was from 2.48% to 5.88%. Samples of 2 - 20 mg. of the acid MPS mixture were used for fractionation.

Fractionation of acid mucopolysaccharides. DEAE-Sephadex A-25 or A-50 was used as described previously [17]. This method permits to estimate the behaviour of the acid MPS in each single aorta. The individual acid MPS were eluted from the Sephadex column by a modified elution system described previously [18,19]. By means of this method the mixture of the acid MPS was separated into 3 fractions: I, hyaluronic acid, eluted with 0.2 M-NaCl - HCl (1:1); II, heparan sulphate, eluted with 0.6 M-NaCl - HCl (1:1); III, chondroitin sulphates, eluted with 1.3 M-NaCl - HCl (1:1). Heparin could be eluted in this system with 2.0 M to 3.0 M-NaCl - HCl as several peaks with variable sulphate content.

Each fraction was analysed for uronic acid according to Dische [8] and with the borate method of Bitter & Muir [6]. For determination of keratan sulphate the modified anthrone method of Holt [13] was used. Sulphate determinations were made according to Antonopoulos [2]. The chondroitin sulphates bearing fraction (1.3 M effluent) was retained for determination of the individual chondroitin sulphate isomers.

Determination of the chondroitin sulphate isomers. The procedure of Schmidt & Dmochowski [19, 20] was used. After enzymic digestion the end products were introduced on a Sephadex G-25 column (2 × 135 cm.) and the undigested dermatan sulphate was removed by gel filtration. Determination of the type of the tetrasaccharides was performed as described by Mathews [15]. Hexosamines were determined as described by Gardell [11].

Standards for paper chromatography. Hyaluronic acid was a preparation of Reanal, Budapest, Hungary. Chondroitin-4-sulphate was purchased from L. Light & Co. Colnbrook, England. Heparan sulphate was kindly given by Prof. Dr. Karl Meyer, Columbia University, New York; another preparation of heparan sulphate was prepared from a heparin side fraction obtained from Upjohn Co., Kalamazoo, U.S.A. Dermatan sulphate was a gift from F. Hoffman-La Roche & Co. Basel, Switzerland.

RESULTS

Total amount of acid mucopolysaccharides in human aorta. From the results shown in Table 1 it may be concluded that the content of acid MPS in human atherosclerotic aorta is increased as compared with that of the newborn or normal adult aorta. The amounts are given in percents of dry, delipidated and decalcified aortic tissue. These results are in good agreement with several other reports [3, 4, 7, 5]. Kaplan & Meyer [14], however, found no difference in the total amount of acid MPS concomitant with age or atherosclerosis. These results, however, concerned non-decalcified human aortae.

Identification of the individual acid mucopolysaccharides. Individual acid MPS were identified by the analytical methods described under Methods and by paper chromatography as hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin-6-sulphate. In all the cases

Table 1

Total acid mucopolysaccharides in normal and atherosclerotic human aortae

The percentage content of acid MPS was determined in dry, delipidated and decalcified aortic tissue. Nitrogen content was determined in the isolated acid MPS mixture. Mean values, \pm S.D., are given.

Group	No. of aortae	Acid MPS as uronic acid (%)	N content in acid MPS (%)
I, Newborn	10	2.45 \pm 0.31	3.65
II, Normal, no medial calcium	15	2.50 \pm 0.37	3.30
III, Normal, medial calcium	13	2.68 \pm 0.44	3.70
IV, Slight atherosclerotic alterations	10	2.88 \pm 0.36	4.55
V, Distinct atherosclerotic alterations	11	3.22 \pm 0.58	3.60
VI, Profound atherosclerotic alterations	31	3.48 \pm 0.65	3.35

Table 2

Analytical data for the separated fractions

Fraction	Hexosamine (%)	Hexosamine		Uronic acid (%)	Sulphate (%)	Ratio: E Dische to E borate
		galactosamine (%)	glucosamine (%)			
I, Hyaluronic acid	38.8	4.1	95.9	36.7	0.2	2.08
II, Heparan sulphate	29.6	0	100	35.5	10.5	1.70
III, Chondroitin sulphate	27.3	100	0	29.6	6.6	

investigated heparin, keratan sulphate, pure chondroitin or chondroitin-4-sulphate were absent. The analytical values for the separate fractions found in an adult aorta are listed in Table 2; other determinations gave similar results. The ratio of extinction for uronic acid in the Dische method (E Dische) to the extinction in the borate method (E borate) was additionally used to characterize the individual compounds.

Behaviour of the acid mucopolysaccharides in atherosclerosis. Fig. 1 shows the relation between the percentage content of the individual acid MPS and age (the total amount of the acid MPS isolated from the aorta was taken as 100%). This group includes normal and only slightly

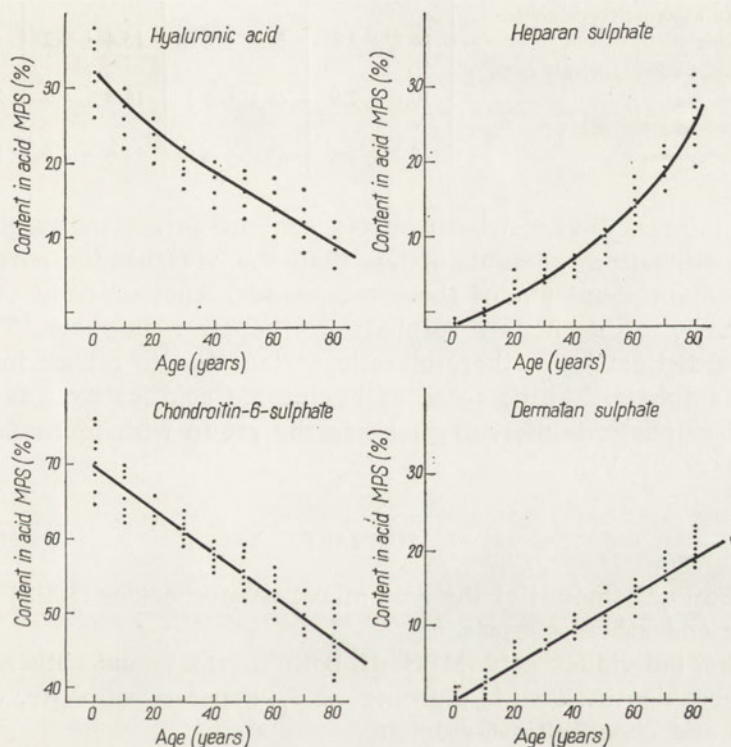


Fig. 1. The relation between the acid mucopolysaccharides and age.

atherosclerotic aortae. Hyaluronic acid and chondroitin-6-sulphate decreased appreciably with age, while dermatan sulphate and heparan sulphate increased considerably. These results are somewhat similar to those of Kaplan & Meyer [14]. Table 3 gives the percentage content of the four individual acid MPS (the total amount of acid MPS isolated in each case was taken as 100%) in normal human aortae and in those with various degrees of atherosclerosis. Table 3 represents the mean values of the individual determinations made separately on each aorta.

Table 3

Percentages of individual acid mucopolysaccharides in normal and atherosclerotic human aortae

Mean values, \pm S.D., are given

Group	Hyaluronic acid (%)	Chondroitin-6-sulphate (%)	Dermatan sulphate (%)	Heparan sulphate (%)
I, Newborn	31.0 \pm 4.7	67.8 \pm 7.1	<1	<1
II, Normal, no medial calcium	28.1 \pm 3.4	66.1 \pm 5.5	3.4 \pm 1.5	2.1 \pm 0.9
III, Normal, medial calcium	21.2 \pm 4.0	61.9 \pm 5.3	9.9 \pm 2.3	7.0 \pm 2.0
IV, Slight atherosclerotic alterations	14.2 \pm 3.8	52.6 \pm 4.8	15.4 \pm 3.2	17.8 \pm 2.6
V, Distinct atherosclerotic alterations	10.8 \pm 2.9	48.1 \pm 6.3	18.6 \pm 4.9	25.5 \pm 5.2
VI, Profound atherosclerotic alterations	8.3 \pm 3.8	43.5 \pm 6.9	21.6 \pm 4.6	26.6 \pm 7.0

It is of interest that newborn aortae contain dermatan sulphate and heparan sulphate in amounts of less than 1%, whereas the normal adult aortae contain about 6% of these compounds. Another point of interest is the ratio of dermatan sulphate to heparan sulphate. This ratio decreases distinctly in atherosclerotic aortae, due to higher increase of heparan sulphate. The decrease of hyaluronic acid, as well as of chondroitin-6-sulphate, is most dramatic in the group with profound atherosclerosis (group VI).

SUMMARY

1. The total amount of the acid mucopolysaccharides (MPS) increases with age and atherosclerosis.
2. The individual acid MPS of both normal and atherosclerotic aorta were identified as hyaluronic acid, heparan sulphate, dermatan sulphate and chondroitin-6-sulphate.
3. Dermatan sulphate and heparan sulphate increase significantly with age and particularly in advanced atherosclerosis. Simultaneously, hyaluronic acid and chondroitin-6-sulphate decrease markedly.
4. Heparin, keratan sulphate, chondroitin and chondroitin-4-sulphate were always absent.

REFERENCES

- [1] Altschuler C. H. & Angewine D. M. - *Amer. J. Pathol.* **27**, 141, 1951.
- [2] Antonopoulos C. A. - *Acta Chem. Scand.* **16**, 1520, 1962.
- [3] Bertelsen Sv. - *Nature* **187**, 411, 1960.
- [4] Bertelsen Sv. & Jensen C. E. - *Acta Pharmacol. Toxicol.* **16**, 250, 1960.

- [5] Bertelsen Sv. & Marcker K. - *Acta Pharmacol. Toxicol.* **18**, 1, 1961.
- [6] Bitter T. & Muir H. M. - *Analyt. Biochem.* **4**, 330, 1962.
- [7] Buddecke E. - *Z. physiol. Chemie* **318**, 33, 1960.
- [8] Dische Z. - *J. Biol. Chem.* **167**, 189, 1947.
- [9] Dyrbye M. & Kirk J. E. - *J. Gerontol.* **12**, 20, 1957.
- [10] Dyrbye M. & Kirk J. E. - *J. Gerontol.* **12**, 23, 1957.
- [11] Gardell Sv. - *Acta Chem. Scand.* **7**, 207, 1953.
- [12] Holle G. & Siedschlag W. D. - *Acta Histochem.* **5**, 369, 1958.
- [13] Holt C. v. - *Klin. Wchschr.* **32**, 661, 1957.
- [14] Kaplan D. & Meyer K. - *Proc. Soc. Exp. Biol. Med.* **105**, 78, 1960.
- [15] Mathews M. B. & Inouye M. - *Biochim. Biophys. Acta* **53**, 509, 1961.
- [16] Meyer K., Davidson E. A., Linker A. & Hoffman P. - *Biochim. Biophys. Acta* **21**, 506, 1956.
- [17] Schmidt M. - *Biochim. Biophys. Acta* **63**, 346, 1962.
- [18] Schmidt M., I Krajowy Kongres Biochemii, Łódź 1963. Abstr. Comm. p. 16.
- [19] Schmidt M. & Dmochowski A., I Krajowy Kongres Biochemii, Łódź 1963. Abstr. Comm. p. 102.
- [20] Schmidt M. & Dmochowski A. - *Biochim. Biophys. Acta* **83**, 137, 1964.
- [21] Taylor H. E. - *Amer. J. Pathol.* **29**, 871, 1953.
- [22] Waters L. L. - *Yale J. Biol. Med.* **29**, 9, 1957.

KWAŚNE MUKOPOLISACHARYDY LUDZKIEJ TĘTNICY ZDROWEJ I MIAŻDŻYCOWEJ

Streszczenie

Oznaczono ilościowo kwaśne mukopolisacharydy w 90 pojedynczych tętnicach ludzkich zdrowych oraz z różnym nasileniem zmian miażdżycowych. Wykazano, że w przypadku starych tętnic, jak również i w przebiegu miażdżycy całkowita ilość kwaśnych mukopolisacharydów jest znacznie podwyższona. Poszczególne kwaśne mukopolisacharydy zidentyfikowano jako: kwas hialuronowy, siarczan heparanu, siarczan dermatanu oraz chondroityno-6-siarczan. W procesie starzenia, szczególnie jednak w przebiegu miażdżycy, następuje silny wzrost siarczanu dermatanu oraz siarczanu heparanu. Jednocześnie obserwuje się znamienny spadek kwasu hialuronowego oraz chondroityno-6-siarczanu. Nie stwierdzono obecności heparyny, chondroityny, siarczanu keratanu ani chondroityno-4-siarczanu.

Received 11 January 1964.

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K. MURAWSKI and K. ZAKRZEWSKI

SULPHYDRYL GROUPS AND HAEM-GLOBIN LINKAGE

Institute of Haematology and Institute of Nuclear Research, Warszawa

The fundamental property of haemoglobins is their ability to combine reversibly with oxygen without a change in the valency state of its iron. This property is wholly dependent upon the protein moiety of a haemoglobin: haem alone is rapidly oxidized to haematin just as is the complex between haem and denatured globin.

The nature of the linkage between haem and globin is not completely understood. The covalent bond that exists between haem and histidine is not the only bond which joins haem and globin, and alone it cannot bring about the stability of divalent iron towards oxygen. The multiplicity of bonds between globin and haem is indicated by several findings: denatured globin is able to bind up to 16 haems per molecule [9]; globin can bind iron-free porphyrins [15]; recent crystallographic data show that there are at least four points of contact between haem and globin [17, 20, 23].

The existence of a bond between carboxyl group of propionyl side chain of haem and globin has been suggested [21, 22] on the basis of the experiments in which etioporphyrin III was coupled to globin. The participation of vinyl side chains in the formation of a bond to globin was indicated by several authors. Deuterohaemin which lacks vinyls, when coupled with native globin forms a haemoglobin capable of reversible binding of oxygen [27]. Removal of vinyls or their replacement by ethyl groups considerably decreases the rate of combination of haem with globin, and protohaem can displace deutero- and mesoporphyrins from their complexes with globin [12].

Which specific group, if any, of globin reacts with the haem vinyls is largely a matter of speculation. It has been suggested that the conformation of the polypeptide chains is complementary to haem and that weak bonds between the two obtain [14]. Indeed, the immediate surrounding of the vinyl side of haem is favourable to formation of hydrophobic bonds [12].

More specifically, the existence of a weak bond between -SH groups

and vinyls was postulated [3] on the basis of the influence of *N*-ethylmaleimide upon the redox potential of haemoglobin. A similar conclusion may be drawn from the fact that blocking of -SH groups decreases the haem-haem interaction [26], comparably to what follows the removal or replacement of vinyl groups [27].

If -SH groups are involved in binding haem with globin, then the formation of a mercaptide may be expected to weaken the coupling between haem and globin. The present experiments were designed to find whether the interaction of oxyhaemoglobin with mercuric ions is followed by increased rates of oxidation of haemoglobin as well as of haem-globin dissociation.

EXPERIMENTAL

Materials and methods

Haemoglobin solutions were prepared from washed human erythrocytes by the toluene method [10]. In several experiments human haemoglobin, crystallized from phosphate buffer [11], was used but the results were essentially the same as with non-crystalline material. The concentration of haemoglobin was estimated by converting it into cyanmethaemoglobin [13] and measuring the extinction at 540 m μ ; the millimolar extinction coefficient of cyanmethaemoglobin was taken as 46.0 for molecular weight 66 800 [6].

The experiments have been carried out in a solution of oxyhaemoglobin in dilute pH 4.5 or pH 4.0 acetate buffer; final concentration of haemoglobin was about 0.15 mM, and the final concentration of acetate in all experiments was 0.067 M. Mercuric ions were added as 1 mM-mercuric acetate in 0.1 M-acetate buffer of pH 4.0 or 4.5.

The progress of the reaction was followed by measuring extinction at 630 m μ , the samples having been incubated at room temperature for appropriate period (see Results). The fraction of oxyhaemoglobin remaining at any time was calculated from the equation:

$$\% \text{HbO}_2 = \left(1 - \frac{E_t - E_0}{E_k - E_0} \right) \times 100$$

in which E_t is the extinction at 630 m μ after a time t , E_0 is the extinction at 630 m μ at $t = 0$, E_k is the extinction at 630 m μ after oxyhaemoglobin was completely converted into methaemoglobin with potassium ferricyanide (for the reaction at pH 4.5) or by treatment with 0.1 N-HCl (for the reaction at pH 4.0). Optical measurements were taken with SF-4 (USSR) spectrophotometer; for some experiments SP-500 (Unicam) instrument was employed.

RESULTS

The product of the reaction at pH 4.5. Oxyhaemoglobin in solution is slowly oxidized with atmospheric oxygen to methaemoglobin [5]. The rate of this reaction is very much increased in the presence of mercuric ions. The validity of the conclusions is very much dependent upon whether the same products are formed in the presence as in the absence of mercury. Fig. 1 shows that the position of all principal bands is the

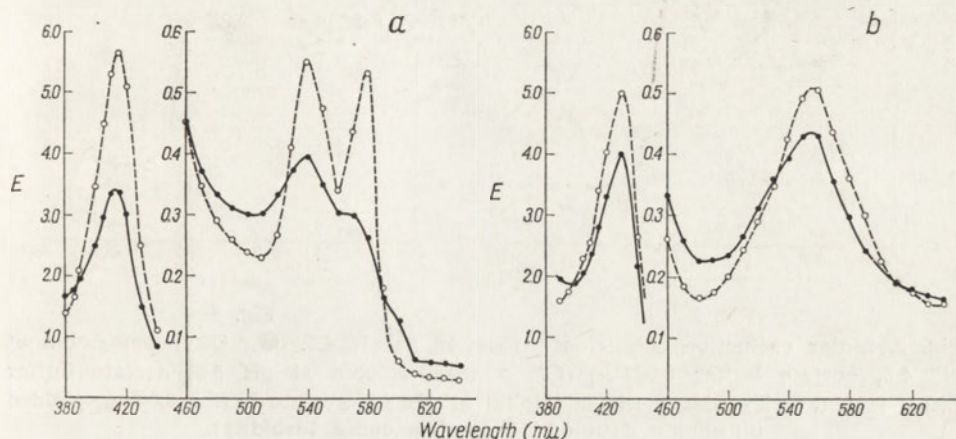


Fig. 1. Oxidation of oxyhaemoglobin and reduction of the oxidized product. (a), Oxidation by atmospheric oxygen at pH 4.5, acetate buffer 0.067 M for 3 hr. at room temp.; (●), in the presence of 4 M Hg²⁺/M haemoglobin; (○), without Hg²⁺. (b), Reaction products as in Fig. 1a, after reduction by dithionite in glycine buffer, pH 7.7.

same, and that both reaction mixtures can be reduced (with crumbs of dithionite under paraffin) into haemoglobin (Fig. 1b). The differences in intensities (Fig. 1a) of corresponding absorption bands result from more extensive conversion of oxyhaemoglobin into methaemoglobin in the presence of mercury than in its absence.

Spectral absorption curves have been computed on the basis of the extinction coefficients shown in Fig. 2. As it may be seen from Fig. 3, the computed curve fits satisfactorily into an experimental one. The deviations which appear at wavelengths below 530 mμ might be due to unapplicability of Lambert-Beer law in that region, and/or to uncertainty of the employed extinction coefficients (ϵ of methaemoglobin at pH 5.0, see legend to Fig. 2).

The product of the reaction at pH 4.0. A rapid splitting of oxyhaemoglobin occurs at pH 4.0. The rate of this reaction is further increased in the presence of mercuric ions. The spectral absorption curve of product formed at pH 4.0 is less distinct than that at pH 4.5 (Fig. 4).

The characteristic peaks of oxyhaemoglobin within visible range disappear, the Soret band becomes diffuse and is shifted towards shorter wavelengths. The exposure of oxyhaemoglobin to 0.1 N-HCl for a period of several hours at 0° brings about more extensive changes throughout

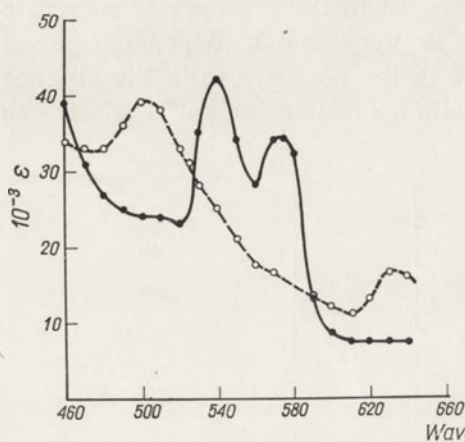


Fig. 2

Fig. 2. Molar extinction coefficients, reaction at pH 4.5. (●), Oxyhaemoglobin at pH 4.5, acetate buffer 0.067 M; (○), methaemoglobin at pH 5.5, acetate buffer 0.02 M [18]. (Ferricyanide methaemoglobin at pH 4.5, acetate buffer 0.067 M, yielded unreliable data owing to developing turbidity).

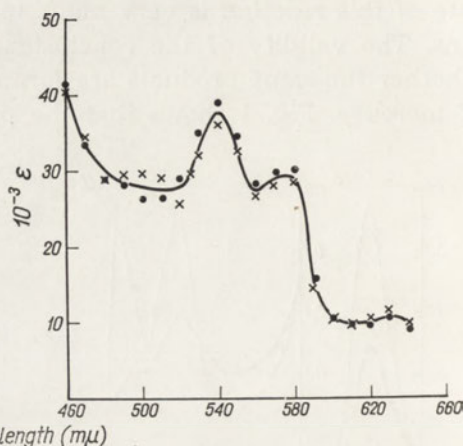


Fig. 3

Fig. 3. Experimental and computed spectral absorption curves. (●), Oxyhaemoglobin oxidized as in Fig. 1a, but for 2 hr.; (×), computed points for the mixture of 60% oxyhaemoglobin and 40% methaemoglobin, based on figures taken from Fig. 2.

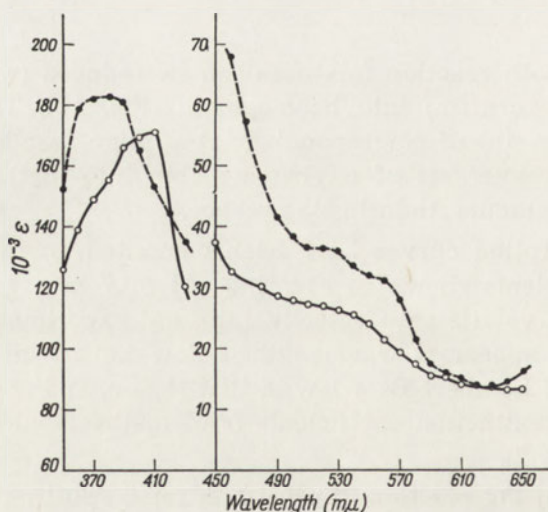


Fig. 4. Molar extinction coefficients, reaction at pH 4.0. (○), Oxyhaemoglobin exposed to pH 4.0 acetate buffer 0.067 M for 1 hr. at room temp.; (●), oxyhaemoglobin exposed to 0.1 N-HCl for 24 hr. at 0°.

the curve, in part certainly due to secondary changes within protein moiety. However, at around 630 m μ , the molar extinction coefficients do not appear to be seriously affected by these secondary changes (Fig. 4).

As it has been well known for many years [2], the acid-denatured haemoglobin can be "re-natured" by neutralization and reduction of the resulting haemichrome. In case of oxyhaemoglobin, the extent of renaturation is about 70%, in case of haemoglobin it is up to 90% [19]. When this was attempted with oxyhaemoglobin split in the presence of mercuric ions, the protein immediately and completely precipitated indicating an irreversible alteration of globin. A control experiment, i.e. the product of the reaction carried on for 3 hr. at pH 4.0, 0.067 M-acetate buffer, room temperature, without mercuric ions, yielded a correct spectrum of haemoglobin, after neutralization and reduction with dithionite.

The kinetics of the reaction at pH 4.5. Solutions of oxyhaemoglobin were prepared with various concentrations of mercuric ions, all in pH 4.5 acetate buffer 0.067 M, and placed in spectrophotometer cuvettes. The reaction was followed by reading the extinctions at 630 m μ at various times. The results are shown in Fig. 5. Clearly, the rate of the oxidation

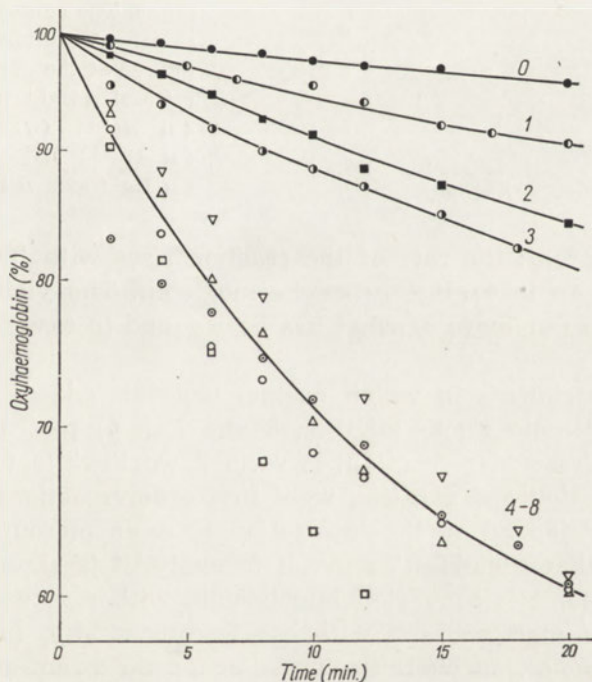
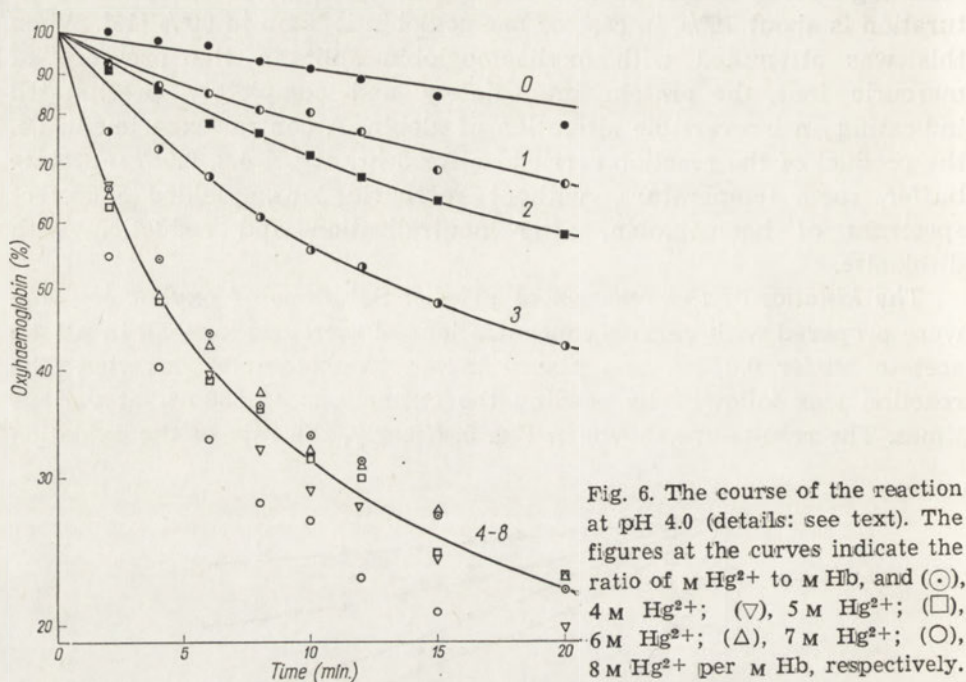


Fig. 5. The course of the reaction at pH 4.5 (details: see text). The figures at the curves indicate the ratio of M Hg²⁺ to M Hb, and (○), 4 M Hg²⁺; (▽), 5 M Hg²⁺; (□), 6 M Hg²⁺; (Δ), 7 M Hg²⁺; (○), 8 M Hg²⁺ per M Hb, respectively.

is directly dependent on the mercury to haemoglobin ratio up to 4 atoms of mercury per one molecule of oxyhaemoglobin. A further increase of this ratio does not influence markedly the rate of the reaction.

The kinetics of the reaction at pH 4.0. The experiments were carried out as described above for pH 4.5 except that pH was 4.0. The results are shown in Fig. 6. An effect qualitatively similar to that observed at



pH 4.5 is apparent: the rate of the reaction rises with increasing Hg^{2+} concentration up to $4 \text{ M Hg}^{2+}/\text{M oxyhaemoglobin}$. The yield of the final product is twice or more of what has been found in case of the reaction at pH 4.5.

In the experiments in which no mercury was added, the points on the semilogarithmic graph (cf. Fig. 5 and Fig. 6) plot a straight line indicating a first order reaction. It seemed worthwhile to assume that both the oxidation and splitting were first order reactions and that the rate constants depend on the interaction between mercury and protein moiety of oxyhaemoglobin. To put it formally, it is assumed that each of the four haems is converted into haemin with a rate constant k_0 in the absence of mercury, and with a rate constant k_{Hg} in the presence of mercury. Hence, an overall expression for the haem-globin dissociation in the absence of mercury is

$$f(t) = A \left(e^{-k_0^{\text{I}} t} + e^{-k_0^{\text{II}} t} + e^{-k_0^{\text{III}} t} + e^{-k_0^{\text{IV}} t} \right)$$

in which A is the fraction of haemoglobin molecule comprising one haemoglobin subunit (i.e. one-quarter molecule), and the superscripts I, II, ... IV denote one of the four haems. In the presence of mercuric ions, a following set of equations obtains:

$$\begin{aligned} f_{1\text{Hg}}(t) &= A \left(e^{-k_{\text{Hg}}^{\text{I}} t} + e^{-k_0^{\text{II}} t} + \dots e^{-k_0^{\text{IV}} t} \right) \\ f_{2\text{Hg}}(t) &= A \left(e^{-k_{\text{Hg}}^{\text{I}} t} + e^{-k_{\text{Hg}}^{\text{II}} t} + e^{-k_0^{\text{III}} t} + e^{-k_0^{\text{IV}} t} \right) \\ f_{3\text{Hg}}(t) &= A \left(e^{-k_{\text{Hg}}^{\text{I}} t} + e^{-k_{\text{Hg}}^{\text{II}} t} + e^{-k_{\text{Hg}}^{\text{III}} t} + e^{-k_0^{\text{IV}} t} \right) \\ f_{4\text{Hg}}(t) &= A \left(e^{-k_{\text{Hg}}^{\text{I}} t} + e^{-k_{\text{Hg}}^{\text{II}} t} + e^{-k_{\text{Hg}}^{\text{III}} t} + e^{-k_{\text{Hg}}^{\text{IV}} t} \right) \end{aligned}$$

The subscripts at $f(t)$: 1Hg, 2Hg, 3Hg, 4Hg denote the reaction carried out in the presence of 1 M Hg, 2 M Hg, 3 M Hg and 4 M Hg per M oxy-haemoglobin, respectively.

In order to find the numerical values for k_0 and for k_{Hg} an XYZ computer has been employed. The least squares method has been tried, but led to ambiguous results. Therefore the successive approximation method was resorted to. It is apparent from Fig. 7 that the solutions

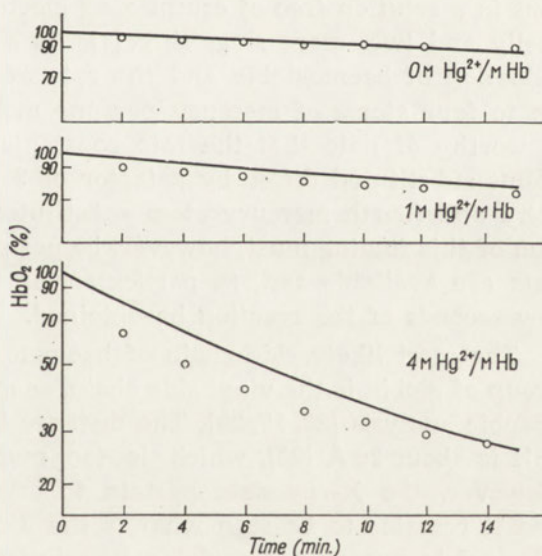


Fig. 7. Reaction kinetics at pH 4.0 (details: see text). (O), Experimental points; (—), computed curve.

are not quite satisfactory, but it is of interest to note (see Table 1) that the best fit has been obtained with three sets of figures, with nearly one order of magnitude difference between k_0 and k_{Hg} values.

Table 1

Rate constants for the splitting of haem from oxyhaemoglobin at pH 4.0

k	Without Hg^{2+} (k_0)	With Hg^{2+} (k_{Hg})
k	0.0087	—
k^I	—	0.051
k^{II}	—	0.068
k^{III}	—	0.150
k^{IV}	—	0.200

DISCUSSION

The results presented above can be accounted for by assuming that mercuric ions are inserted between sulphhydryl group of globin and haem. There are six cysteine residues in human haemoglobin [8]. Their reactivity has been studied extensively in recent years and it was concluded that only two of them are "reactive" towards various sulphhydryl reagents [1]; they appear to be located in the β -chain [25]. However, which -SH groups in haemoglobin are reactive, depends on the kind of reagent employed for their detection. With organic mercurial, the two groups in the β -chain are reactive but with inorganic mercuric ions in a solution free of complexing electrolytes, four sulphhydryls react easily and two, more sluggishly [7]. As shown above, both the rate of oxidation of haemoglobin and the rate of its splitting increase steadily up to four atoms of mercury per one molecule of haemoglobin, and it is worthy of note that the rate constants for the first two mercury atoms substituted differ by a factor of 3 to 4 from the factors for the third and fourth mercury atom substituted. A more detailed consideration of this finding must, however, be postponed until a more satisfactory data are available and, in particular, until the rate constants for first few seconds of the reaction be obtained.

The most likely side chain of haem to form a link with sulphhydryl group of globin is the vinyl side chain, as may be deduced from crystallographic analysis [23, 17, 20]. The distance between haem and the nearest -SH is about 20 Å [23], which is too much for any kind of a bond. However, the X-ray data pertain to crystalline state of haemoglobin and it remains to be seen what is the distance between vinyl and sulphhydryl in human haemoglobin in solution.

We suggest that a labile link forms between the vinyl side chain of haem and sulphhydryl group of globin, perhaps by making use of the two available electrons on sulphur atom. On approach of oxygen, the electrons are pulled towards the iron-oxygen complex through the

double bond system of porphyrin ring. Thus, a second junction, in addition to iron-histidine one, is established between haem and a system of double bonds of polypeptide chain(s). In such a way, a large ring-like structure would be instantaneously formed, and likely to be easily destroyed by formation of a mercaptide.

There are several consequences of this hypothesis as to the structural basis of haemoglobin function. The haem-haem interaction has already been shown to depend upon the -SH groups [24] and appears to be absent [16] or at most a very weak one [28] in myoglobins which do not contain sulphydryl groups. This interaction would appear to be channeled through the vinyl -SH bond. The Bohr effect which is attributed by some authors [24] but not by others [4] to -SH groups, would result from a decreased attraction of proton by sulphur atom due to the electron shift, within the postulated bond, during oxygenation. The vinyl-sulphydryl bond will increase the stability of divalent iron towards oxidation by bringing into action many more π -electrons. The same mechanism is bound to augment the forces which hold haems to globin.

The authors gratefully acknowledge the assistance of Mr. B. Ostalski of the Institute of Mathematical Machines, Polish Academy of Sciences for programming and computations on the XYZ computer.

This paper has been supported in part by a grant from the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

SUMMARY

A study was made of oxyhaemoglobin solution in dilute buffers pH 4.5 and pH 4.0 exposed to atmospheric oxygen in the presence and in the absence of inorganic mercuric ions. It has been found that mercury strongly accelerated the splitting of haemoglobin at pH 4.0, and its auto-oxidation at pH 4.5; the rate constants were raised by about one order of magnitude.

The hypothesis is proposed that a labile vinyl-sulphydryl bond obtains in oxyhaemoglobin, which strengthens the haem-globin linkage and increases the stability of divalent iron by bringing into action double bonds of polypeptide chain(s).

REFERENCES

- [1] Allison A. C. & Cecil R. - *Biochem. J.* **69**, 27, 1958.
- [2] Anson M. L. & Mirsky A. B. - *J. Gen. Physiol.* **9**, 169, 1925.
- [3] Behlke J. & Scheler W. - *Naturwiss.* **48**, 717, 1961.
- [4] Benesch R. & Benesch R. E. - *J. Biol. Chem.* **236**, 405, 1961.
- [5] Brooks J. - *Proc. Roy. Soc. B, London* - **109**, 35, 1931.

- [6] Cannan R. K. - *Science* **127**, 1376, 1958.
- [7] Cecil R. & Snow N. S. - *Biochem. J.* **82**, 247, 1962.
- [8] Cole R. D., Stein W. H. & Moore S. - *J. Biol. Chem.* **233**, 1359, 1958.
- [9] Drabkin D. L. - *J. Biol. Chem.* **158**, 721, 1945.
- [10] Drabkin D. L. - *J. Biol. Chem.* **164**, 703, 1946.
- [11] Drabkin D. L. - *J. Biol. Chem.* **185**, 231, 1950.
- [12] Gibson Q. H. & Antonini E. - *J. Biol. Chem.* **238**, 1384, 1963.
- [13] Green P. & Teal C. F. J. - *Am. J. Clin. Path.* **32**, 216, 1959.
- [14] Haurowitz F. & Hardin R. L., in *The Proteins* (H. Neurath & K. Bailey, eds.) Vol. II, p. 279; Academic Press, New York, 1954.
- [15] Hill R. & Holden H. F. - *Biochem. J.* **20**, 1326, 1926.
- [16] Kaziro K. & Tsuschima K. in *Haematin Enzymes* (J. E. Falk, R. Lemberg & R. K. Morton, eds.) p. 80; Pergamon Press, Oxford 1961.
- [17] Kendrew J. C., Watson H. C., Strandberg B. E., Dickerson R. E., Phillips D. C. & Shore V. C. - *Nature* **190**, 666, 1961.
- [18] Kubowitz F. - *Z. ges. inn. Med.* **501**, 1948.
- [19] Lemberg R. & Legge J. W. - *J. Proc. Roy. Soc. N. S. Wales* **72**, 62, 1938.
- [20] Muirhead H. & Perutz M. F. - *Nature* **199**, 633, 1963.
- [21] O'Hagan J. E. - *Nature* **184**, 1808, 1959.
- [22] O'Hagan J. E. - *Biochem. J.* **74**, 417, 1960.
- [23] Perutz M. F., Rossman M. G., Cullis A. F., Muirhead H., Will G. & North A. C. T. - *Nature* **185**, 416, 1960.
- [24] Riggs A., in *Sulfur in Proteins* (R. Benesch, R. E. Benesch, P. B. Boyer, I. M. Klotz, W. R. Middlebrook, A. G. Szent-Györgyi & D. R. Schwartz, eds.) p. 173; Academic Press, New York, 1959.
- [25] Riggs A. & Wells M. - *J. Biol. Chem.* **236**, 1948, 1961.
- [26] Riggs A. & Wolbach R. A. - *J. Gen. Physiol.* **39**, 585, 1956.
- [27] Rossi-Fanelli A. & Antonini E. - *Arch. Biochem. Biophys.* **80**, 308, 1959.
- [28] Theorell H. - *Biochem. Z.* **268**, 55, 1934.

GRUPY SULFHYDRYLOWE A WIAZANIE MIĘDZY HEMEM I GLOBINĄ

Streszczenie

Badano zachowanie się oksyhemoglobiny w rozcieńczonych buforach pH 4.5 i pH 4.0, w kontakcie z tlenem atmosferycznym, w obecności i w nieobecności nieorganicznych jonów rtęciowych. Stwierdzono, że jony rtęciowe silnie przyspieszają rozszczepianie hemoglobiny w pH 4.0 i jej autooksydację w pH 4.5. Stałe szybkości reakcji rosły o około jeden rząd wielkości.

Postawiono hipotezę, że w oksyhemoglobinie istnieje labilne wiązanie pomiędzy resztą winylową a grupą sulfhydrylową, które wzmacnia wiązanie hemu z globiną i wzmacnia stabilność dwuwartościowego żelaza przez wprowadzenie do działania wiązań podwójnych łańcucha (lub łańcuchów) polipeptydowych.

Received 13 January 1964.

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**FORMATION OF INDOLE-PYRIDOXAL COMPLEX
IN THE URINE OF SCHIZOPHRENICS***Brain Research Institute, Medical University, Szeged, Hungary*

In our work concerning the pathological processes in schizophrenia we have studied for many years the disturbances of tryptophan metabolism. We examined the urine excretion of indole compounds by patients in different phases of schizophrenia maintained on a protein-poor diet and also after tryptophan loads (Huszák & Durkó, 1961, 1962, 1963). In these experiments, freshly voided samples were immediately adjusted with hydrochloric acid to pH 3. The urine collected during 24 hr. was extracted with chloroform and from the aqueous phase the indole compounds were isolated; it was then observed that the discarded chloroform phase turned red after a certain time. The red pigment of the chloroform phase was also examined and the results are described in the present paper.

Nencki & Sieber (1862) were the first to observe the formation of the red colour in the urine after addition of hydrochloric acid. They termed this red pigment uroroseine. Herter (1908) established that uroroseine is formed from indole acetic acid, probably as its oxidation product. This assumption was supported by Dobeneck *et al.* (1956) who reported the uroroseine reaction of indole acetic acid. A good review of the literature concerning uroroseine was published recently by Ressel (1960).

Ross (1913) observed a positive uroroseine reaction in different mental diseases, including schizophrenia. Armstrong & Robinson (1954) observed in phenylketonuria a positive uroroseine reaction as well as large amounts of indole acetic acid. Jepson (1956) found that increased indole acetic acid excretion is accompanied by uroroseine. According to Armstrong (1958) the positive uroroseine reaction may also be observed in the urine of normal subjects.

Scott (1961) observed that in acid medium indole and pyridoxal phosphate (or pyridoxal) react to form a red pigment. The formation of the pigment could be prevented by dimedone which formed a compound with pyridoxal.

The above data seemed to suggest that the red compound formed in the urine of schizophrenics at pH 3 may be an indole-pyridoxal complex. To check this supposition the following experiments were performed.

METHODS

The urine adjusted with hydrochloric acid to pH 3 was shaken with chloroform. The chloroform phase was centrifuged and the red pigment was extracted with amylalcohol. Then the solvent was evaporated and the red residue obtained was well soluble both in methanol and water. For spectrophotometric examinations (Hilger H 700, Uvispek) the pigment dissolved in methanol was used. For comparison, an indole-pyridoxal compound was synthesized. For the synthesis, indole acetic acid, physiologically present in urine, was used. A mixture of equal volumes of 0.1 M-indole acetic acid and 0.1M-pyridoxal phosphate was adjusted with hydrochloric acid to different pH values. The absorption spectra of the

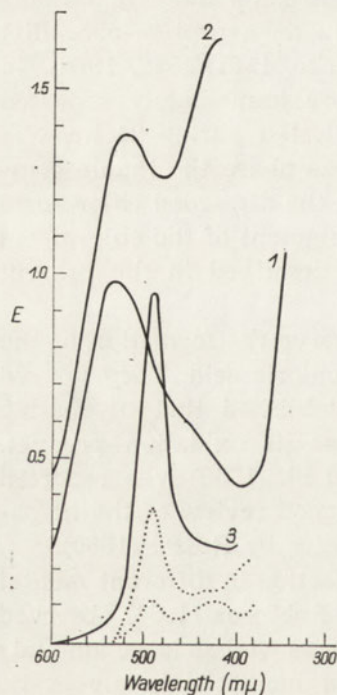


Fig. 1. The absorption spectra (—), of the indole acetic acid - pyridoxal complex at pH 1, 2 and 3, and (....), of the red pigment extracted from urine at pH 3.

formed coloured compounds at different pH values and of the red pigment extracted from the urine, are shown in Fig. 1; it may be seen that the absorption spectrum of the red pigment from the urine is the same as that of the indole-pyridoxal complex obtained at pH 3.

If before the acidification of the urine, dimedone was added the red pigment was not formed. In control urines, in which the red compound

did not form, the addition of indole acetic acid and pyridoxal before the chloroform extraction resulted in the appearance of the red colour.

Experiments were also performed on healthy persons and schizophrenic patients in whose urine the red pigment did not form. They were loaded intravenously 3 times daily with pyridoxal (vitamin B₆), and on the next day the appearance of the red colour in the chloroform phase of the urine was observed. When to a patient maintained on a protein-low diet and treated with pyridoxal, tryptophan was also given *per os*, the red colour appeared in the chloroform phase.

These results indicate that the red pigment formed in the chloroform extract of acidified urine of schizophrenic patients is the indole pyridoxal complex described by Scott (1961). We expect to be able to confirm this supposition by analytical methods and infra-red examinations which are now in progress.

Table 1

Formation in the urine of the red pigment in different diseases

Type of disease	No. of cases	No. of examinations	Positive	Negative
Schizophrenia	16	68	55	13
Depression	4	11	3	8
<i>Delirium tremens</i>	2	2	2	—
Epilepsy	2	2	1	1
Lesion of the liver	2	2	2	—
Other diseases	3	4	—	4

It should be noted that the appearance of the red pigment is not specific for schizophrenia as it occurs, though far less frequently, in the urine of other psychotics (Table 1).

DISCUSSION

The red compound isolated from the urine of schizophrenics has not been yet fully identified, but nevertheless the presented results of experiments carried out *in vitro* and *in vivo* support the supposition that it does arise from indole and pyridoxal. This compound cannot be identical with nephroroseine described by Arnold (1909, 1911) which gave a positive uroscopine reaction. Nephroroseine was identified by Kimming *et al.* (1958) and confirmed by Jepson & Spiro (1960) as *N*-[β -(indolyl)-(3-acrylyl)-glycine. Mellman *et al.* (1963) described the chromatographic characteristics of this compound which gave an orange spot after treatment with 25% HCl. Its E_{max} was at 509 m μ . On administration of L-trypt-

tophan its amount in the urine did not increase. Our pigment had a different absorption maximum and its amount increased after administration of tryptophan and pyridoxal, indicating that the pigment studied is an indole-pyridoxal complex.

The abnormal excretion of tryptophan metabolites in schizophrenia has been known for a long time. Several observations indicate that in schizophrenia some disturbances of phosphopyridoxal-containing enzymes (transaminase, decarboxylase etc.) may be present. Benassi *et al.* (1961) found that the metabolism of kynurenine is disturbed and the accumulation of anthranilic and xanthurenic acids can be observed. It is well known that pyridoxal enzymes are involved in the metabolism of these compounds. Dalglish (1951) observed disturbances of tryptophan metabolism in human subjects after administration of isoniazide, which is an inhibitor of the pyridoxal enzymes. Price (1957) in experiments with humans as well as with animals kept on a pyridoxine-free diet, noted increased excretion of xanthurenic acid which returned to normal levels after administration of pyridoxine. Later Price (1961) established that the deficiency of pyridoxal leads not only to disturbances in the metabolism of xanthurenic acid, but in tryptophan metabolism in general. Caceda (1961) reported that in different types of schizophrenia various amounts of pyridoxal are excreted. He assumes that in the organism of schizophrenics there may be a pyridoxal deficiency.

These data as well as our own observations permit to assume that the disturbance of tryptophan metabolism in schizophrenics may be attributed to disturbances of phosphopyridoxal enzymes.

The demonstration of the indole-pyridoxal complex in the urine of schizophrenic patients on a normal diet indicates that the excretion of pyridoxal and the indole metabolites of tryptophan is in those patients increased over the normal values. It seems probable that the pyridoxal is not utilized as a coenzyme factor which leads to its increased excretion and at the same time causes disturbances in metabolic reactions catalysed by phosphopyridoxal enzymes. As our future task we envisage a study of the causes of non-utilization of pyridoxal by schizophrenics.

SUMMARY

The urine of schizophrenic patients adjusted to pH 3 was extracted with chloroform. In the extract a red compound was formed which could be identified as the indole-pyridoxal complex.

In the urine of normal subjects the red pigment was not formed, but it could be found after loading with tryptophan and pyridoxal.

REFERENCES

- [1] Armstrong M. D. & Robinson K. S. - *Arch. Biochem. Biophys.* **52**, 287, 1954.
- [2] Armstrong M. D. - *J. Biol. Chem.* **232**, 17, 1958.
- [3] Arnold V. - *Z. physiol. Chem.* **61**, 240, 1909; **71**, 3, 1911; cited after Decker P. - *Naturwiss.* **44**, 330, 1957.
- [4] Benassi C. A., Benassi P., Allegri G. & Ballarin P. - *J. Neurochem.* **7**, 264, 1961.
- [5] Caceda G. S. - *Rev. Fac. Farm. Bioquim.* **23**, 77, 1961.
- [6] Dalgliesh C. E., Knox W. E. & Neuberger A. - *Nature* **168**, 20, 1951.
- [7] Dobeneck v. H. - *Z. physiol. Chem.* **304**, 26, 1956.
- [8] Herter C. A. - *J. Biol. Chem.* **4**, 253, 1908.
- [9] Huszák I. & Durkó I., *Proceedings of the Third World Congress of Psychiatry* (Canada 1961) p. 674.
- [10] Huszák I. & Durkó I. - *Psychiat. Neurol.* **143**, 407, 1962.
- [11] Huszák I. & Durkó I., *IVth Congress of Neuro-Psychiatry* (Moscow 1963). In press.
- [12] Jepson J. B. - *Biochem. J.* **64**, 14P, 1956.
- [13] Jepson B. & Spiro J. J., in *The metabolic basis of inherited diseases* (J. B. Stabury, J. B. Wynguarden & D. S. Fredrickson, eds.) p. 1338. McGraw Hill, New York 1960.
- [14] Kimming J., Sticherling W., Tschesche R. & Urbach H. G. - *Z. physiol. Chem.* **311**, 234, 1958.
- [15] Mellman W. J., Barness L. A., Tedesco T. A. & Besselman D. - *Clin. Chim. Acta* **8**, 843, 1963.
- [16] Nencki M. & Sieber I. - *J. prakt. Chem.* **26**, 333, 1862.
- [17] Price J. M. - *J. Clin. Invest.* **36**, 1600, 1957.
- [18] Price J. M. - *Fed. Proc.* **20**, 223, 1961.
- [19] Ressel K. - *Z. inn. Med.* **15**, 203, 1961.
- [20] Ross E. L. - *Arch. Int. Med.* **12**, 112 and 231, 1913.
- [21] Scott T. A. - *Biochem. J.* **80**, 462, 1961.

POWSTAWANIE KOMPLEKSU INDOLO-PIRYDOKSALOWEGO
W MOCZU SCHIZOFRENİKÓW

Streszczenie

Mocz chorych na schizofrenię zakwaszano do pH 3 i ekstrahowano chloroformem. Czerwony związek powstający w ekstrakcie zidentyfikowano jako kompleks pirydoksalu z indolem.

Czerwony barwnik nie powstawał w moczu normalnym, pojawiał się jednak po obciążeniu tryptofanem i pirydoksałem.

Received 14 January 1964.

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ON THE USE OF TANNIN FOR THE PREPARATION OF GLYCOPROTEIN HORMONES FROM HOG PITUITARY GLAND

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It has been shown in this Laboratory [13] that proteins regenerated from protein-tannin complexes by caffeine retained their biological properties. This method was also applied for the preparation of proteins soluble in sulphosalicylic acid (SSA) such as seromucoid and lysozyme [15].

The glycoprotein hormones of the pituitary gland, namely, thyrotropic, luteinizing and follicle-stimulating hormones are known to be soluble in sulphosalicylic and trichloroacetic acids. To purify the thyrotropin Loeser [10], Lambie & Trikojus [8] and Heideman [5] utilized this property but the yield obtained was rather unsatisfactory. Tannin has been applied by Levin & Tyndale [9] for the isolation of gonadotropins from urine. In the present work an attempt was made to find out whether the pituitary glycoprotein hormones could be isolated from SSA-extracts by tannin and caffeine without losing their activity, and whether this will prove to be a satisfactory method for obtaining highly concentrated thyrotropic and gonadotropic hormones. The original hormonal activity assayed in the fresh pituitary gland was compared with that of the final product.

EXPERIMENTAL

Analytical methods. Protein was estimated by the tannin micromethod [11], sialic acid as described by Seifer & Gerstenfeld [17].

Materials. Hog pituitary glands obtained from a slaughterhouse were frozen and stored at -10° for not more than 6 days. From the frozen glands, anterior lobes were separated, washed several times with cold 0.9% NaCl solution until free from blood, blotted with filter paper, weighed and used for extraction.

Extraction with sulphosalicylic acid and preparation of the active material

Frozen anterior lobes were ground at 5° in a mortar for about 10 min. with a double amount of 0.15 M-sulphosalicylic acid. After centrifugation the residue was washed 5 times with 0.15 M-SSA. To the pooled extracts cold water was added to a volume 20 times that of the lobe weight. This solution contained about 0.025 g. of protein per 100 ml. It was found that by 5-fold extraction about 80% of all the protein soluble in SSA were extracted; estimations of the SSA-soluble protein in the anterior pituitary lobes are shown in Table 2.

The extracted proteins were precipitated by tannin and regenerated from the complex by caffeine according to the method of Mejbaum-Katzenellenbogen [12, 14]. The extract was adjusted with 2 N-NaOH to pH 4-5 (yellow-green bromophenol blue) and the proteins were precipitated with tannin (5 mg. tannin/mg. protein). After 20 min. the sample was centrifuged, the protein-free supernatant discarded and the precipitate washed several times with cold distilled water to remove the sulphosalicylic acid. To the sedimented by centrifugation tannin-protein complex, caffeine *in substantia* was added in an amount twice that of the protein and the mixture was stirred with a glass rod for 10 min. Water was then added in such an amount as to give final protein concentration in the mixture of about 2%. After centrifugation the supernatant was withdrawn and the residue was extracted twice with distilled water added in amounts half that added at first. The combined supernatants

Table 1

Regeneration of sulphosalicylic acid-soluble protein with tannin and caffeine

Anterior pituitary lobes were extracted and protein concentrated as described under Experimental.

Weight of sample (g.)	Protein in SSA extract (g./100 ml.)	SSA-soluble protein (g./100 g. tissue wet wt.)	Total amount of extracted protein (g.)	Protein content in regenerated fluid (g./100 ml.)	Protein loss	
					protein in tannin-caffeine pellet (g.)	(%)
19.6	0.032	0.66	0.130	1.02	0.016	12
30.5	0.022	0.43	0.132	1.10	0.025	18
40.0	0.024	0.48	0.192	0.99	0.037	19
100.0	0.027	0.54	0.540	1.60	0.081	15
Mean value		0.53 ± 0.097				

gave a clear, slightly yellow solution. The caffeine present in the solution was removed by freezing and centrifugation of the precipitate. Then the protein solution was lyophilized and stored in an evacuated desiccator over P_2O_5 at 5° . The final product was a white amorphous powder, soluble in water, 0.9% sodium chloride solution, and in 0.15 M-sulphosalicylic acid.

In some lyophilized preparations, estimations of water content were made by drying at 100° over P_2O_5 . After 35 hr. no further decrease in weight was observed. The average values obtained were: 46% of water, 43% of protein and 1.4% of sialic acid.

The extraction with SSA and the efficiency of the tannin-caffeine procedure for regenerating the extracted proteins are presented in Table 1; the results refer to 4 preparations for which different amounts of pituitary anterior lobes were used. From 100 g. of pituitary lobes 0.43 to 0.66 g. of protein was extracted, the concentration of protein in the extract being about 0.026%. After tannin-caffeine procedure the solution contained about 1.2% protein; thus a nearly 50-fold concentration was obtained. Not the whole amount of protein present in the SSA extract was recovered, as about 15% remained in the tannin-caffeine sediment.

Hormonal activity

Thyrotropic activity. The assays were performed by the histological method of Junkman & Schoeller [6] who defined a unit of thyrotropin as the daily amount of hormone required to produce a definite histological response after 3 daily injections in one out of two guinea pigs weighing between 100 and 150 g. Guinea pigs of both sexes from a single source were used. The thyroid epithelium was considered to be definitely stimulated when the acini were lined with cuboidal epithelium, and a lack of response was assumed when the epithelium lining the glands was flat. The International Unit and the USP unit equal 10 units of Junkmann & Schoeller (J.S. units).

To test the activity of the regenerated SSA-soluble protein, this was appropriately diluted with 0.9% NaCl and about 500 μ g. of protein was injected daily into guinea pigs. Thyroids of the injected animals were found to be highly hypertrophic and histological examinations showed an extremely stimulated gland. The minimum effective dose was found to be 10 μ g. of protein and it resulted in a marked increase in the size of the thyroid epithelial cells (Fig. 1). A dose of 5 μ g. was ineffective; thyroid follicles did not differ from those of the control animals (Fig. 2).

The thyrotropic activity of the lyophilized preparations was the same, 10 μ g. of protein being the minimum effective dose. As our lyophilized preparation contained about 50% of protein, its activity may be expressed as 50 J.S. units per mg. (about 5 I.U.). Condliffe & Bates [2]

and Carsten & Pierce [1] purified the thyrotropic activity to a similar degree using cation exchange column chromatography or countercurrent distribution.

Gonadotropic activity. The immature mouse uterus test of Klinefelter *et al.* [7] was used to measure the combined effect of luteinizing and follicle-stimulating hormones. Subcutaneous injections in 5 equal doses for 3 days were given to female mice weighing 8 - 10 g., 5 animals being used for each dose level. The animals were killed 72 hr. after the first injection and the uteri were dissected and weighed. A mouse unit was defined as the minimum total amount of the injected protein which caused a two-fold increase in uterine weight. The minimum effective dose causing such an increase was found to be 30 μ g. of protein. Thus 1 mg. of the concentrated protein had a potency of 33 mouse units. The minimum effective dose of the lyophilized preparation was 40 μ g. of protein.

*Hormonal activity in homogenates of the pituitary gland
and in the final product*

To compare the hormonal activity of the anterior pituitary lobes with that of the final preparation, the content of SSA-soluble protein in the anterior lobes, and the potency of a freshly prepared homogenate were estimated. Anterior lobes were homogenized with 9 vol. of 0.15 M-SSA in a Potter homogenizer and after centrifugation the content of protein in the supernatant was estimated. As shown in Table 2, 100 g. of anterior lobes contained 0.64 g. \pm 0.066 of SSA-soluble protein, so 6 μ g. of this protein were present in 1 mg. of the tissue.

For hormonal potency estimations frozen anterior lobes were homogenized in 0.9% NaCl solution. It was found that 2 - 3 mg. of the tissue contained the minimum effective dose of the thyrotropic hormone. The minimum effective dose of our preparation was 10 μ g. of protein, that is the amount present in 2 mg. of tissue. Thus it can be concluded that all thyrotropic activity present in the lobe was extracted by sulphosalicylic acid and found in our preparation.

The assays of gonadotropic activity in a freshly prepared anterior lobe homogenate showed that 5 mg. of the tissue contained the minimum effective dose. For the final preparation, the minimum effective dose was 30 μ g. of protein, which corresponded to 5 mg. of fresh tissue. Similarly as the thyrotropic activity, also all gonadotropic potency was present in the preparation.

These results indicate that no loss of hormonal activity occurred during the preparation procedure, which was carried out at room temperature. It is known that the purified thyrotropic hormone may be stored at room temperature for some time without loss of activity, but crude preparations are unstable at room temperature. This is assumed to be

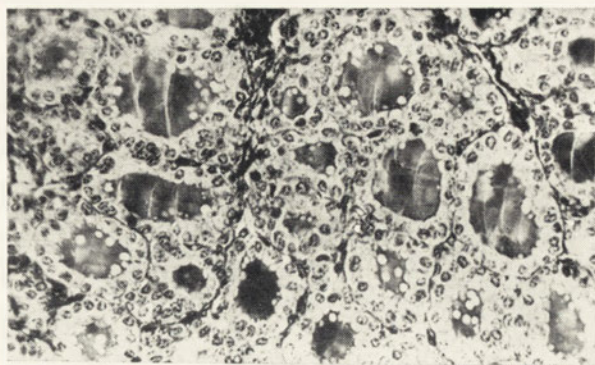


Fig. 1. The stimulated guinea pig thyroid after 3 daily injections of 10 μ g. of the regenerated SSA-soluble protein from the anterior pituitary lobe. ($\times 344$)

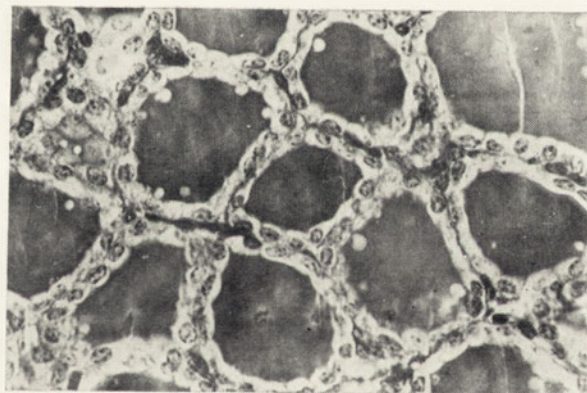


Fig. 2. Thyroid gland of a normal guinea pig. ($\times 344$)

Table 2

Content of sulphosalicylic acid-soluble protein in the anterior pituitary lobes

Anterior lobes were homogenized with 9 vol. of 0.15 M-SSA. After centrifugation, protein was estimated in the supernatant by the tannin micromethod [11]. One g. of the tissue corresponded to 3-4 anterior lobes.

Sample no.	Weight of sample (g.)	Protein content (g./100 g. wet weight)
1	1.0	0.65
2	1.0	0.72
3	1.0	0.70
4	0.5	0.58
5	0.5	0.54
6	0.5	0.65
7	0.5	0.74
8	0.5	0.64
9	0.5	0.54
10	0.5	0.65
11	0.5	0.62
Mean value		0.64 ± 0.066

caused by proteinases which have been claimed to be present in the pituitary gland [4]. The activity of those enzymes in sulphosalicylic acid extracts is hardly to be expected, we have therefore studied the effect of temperature on the activity of the homogenate and our preparation. Homogenates freshly prepared or stored during the 3-day test at room temperature under toluene were assayed for thyrotropic activity. Similarly a preparation stored in a refrigerator and one stored at room temperature were tested. Doses injected were three times the value of the minimum effective dose. It was shown that the homogenate stored at room temperature lost its activity, whereas storing at room temperature had no effect on the potency of the final preparation.

DISCUSSION

These results demonstrate the efficiency of sulphosalicylic acid for direct extraction of the glycoprotein hormones from the anterior pituitary lobe. Sulphosalicylic acid not only protects the hormone from the action of proteolytic enzymes but probably causes as well a dissociation of the active material from inactive protein present in the tissue. It was thus of interest to find whether some adrenotropic activity is present in our final preparation. An assay was made on rats using the thymus involution

test, and adrenotropic activity of 0.85 units/mg. was found¹. Similar results were recently reported by Currie & Davis [3] who used trichloroacetic acid extracts from human pituitary glands for the preparation of corticotropin of low molecular weight.

The yield of the hormone according to our procedure can be calculated from a typical experiment. From 30.5 g. of the anterior lobe tissue having a total thyrotropic potency of 12 000 J.S. units and gonadotropic activity of 6100 mouse units (the minimum effective doses being 2.5 and 5 mg. of tissue, resp.) about 9.5 ml. of concentrated protein solution was obtained with a protein content of 100 mg. and a potency of 10 000 J.S. units and 3300 mouse units. After lyophilization 190 mg. of the preparation was obtained. The yield of the thyrotropic activity corresponded to 75% of the activity present in the anterior lobes. The recovery is even higher if one considers the amount of protein lost in the tannin-caffeine residue.

The thyrotropic potency of the lyophilized preparation was 50 J.S. units per mg. (5 I.U.). Condliffe & Bates [2] described the purification of bovine thyrotropic hormone to a level of 5 I.U./mg. with a 50% yield. Pierce & Nyc [16] obtained a material having the same thyrotropic potency but the yield was only 33% of the activity present in frozen anterior beef pituitary lobes.

The method described in this paper does yield satisfactory amounts of concentrated thyrotropic hormone and gonadotropic hormones in forms readily susceptible to further purification, and the obtained preparation may be used for experimental studies even without further purification.

SUMMARY

A new method is described for the preparation of high potency concentrates of thyrotropic and gonadotropic hormones. Frozen anterior pituitary lobes were homogenized in 0.15 M-sulphosalicylic acid and the extracted protein concentrated by the tannin-caffeine procedure. The regenerated proteins contained all thyrotropic and gonadotropic activities present in the lobes.

REFERENCES

- [1] Carsten M. E. & Pierce J. G. - *J. Biol. Chem.* **229**, 61, 1957.
- [2] Condliffe P. G. & Bates R. W. - *J. Biol. Chem.* **223**, 843, 1956.
- [3] Currie A. R. & Davies B. M. A. - *Acta Endocrin.* **42**, 69, 1963.
- [4] Ellis S. - *J. Biol. Chem.* **235**, 1694, 1960.
- [5] Heideman M. L., Jr. - *Endocrinology* **53**, 640, 1953.

¹ The assay was kindly performed by Mr. St. Południkiewicz from the Pharmacological Laboratory of Jeleniogórskie Zakłady Przemysłu Farmaceutycznego.

- [6] Junkmann K., in *Handbuch der Biologischen Arbeitsmethoden* (E. Abderhalden, ed.) Abt. V, Teil 3B, 1072. Urban & Schwarzenberg, Berlin 1938.
- [7] Klinefelter H. F., Albright F. & Griswood G. C. - *J. Clin. Endocrin.* **3**, 529, 1943.
- [8] Lambie C. G. & Trikojus U. M. - *Biochem. J.* **31**, 843, 1937.
- [9] Levin L. & Tyndale H. - *Proc. Soc. Exp. Biol. N.Y.* **34**, 516, 1936.
- [10] Loeser A. - *Klin. Wochschr.* **11**, 1271, 1932.
- [11] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **2**, 279, 1955.
- [12] Mejbaum-Katzenellenbogen W. - *Acta Biochim. Polon.* **6**, 375, 385, 1959.
- [13] Mejbaum-Katzenellenbogen W. & Morawiecka B. - *Acta Biochim. Polon.* **6**, 453, 1959.
- [14] Mejbaum-Katzenellenbogen W., Morawiecka B. & Dobryszczyka W. - *Acta Biochim. Polon.* **7**, 401, 1960.
- [15] Mejbaum-Katzenellenbogen W., Dobryszczyka W. & Morawiecka B. - *Nature* **192**, 262, 1961.
- [16] Pierce J. G. & Nyc J. F. - *J. Biol. Chem.* **222**, 777, 1956.
- [17] Seifer A. & Gerstenfeld S. - *J. Lab. Clin. Med.* **50**, 17, 1957.

ZASTOSOWANIE TANINY DO PREPARACJI HORMONÓW GLIKOPROTEIDOWYCH Z PRZYSADKI WIEPRZOWYCH

Streszczenie

Opisano nową metodę uzyskiwania preparatów o dużej aktywności tyreotropowej i gonadotropowej. Przednie płaty przysadki homogenizowano z 0.15 M-kwasem sulfosalicylowym. Ekstrahowane białka zagęszczano przy pomocy taniny i kofeiny. Regenerowane białko zawierało całą aktywność tyreotropową i gonadotropową, oznaczaną w homogenatach z przedniego płata.

Received 23 January 1964.

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THE INTERACTION OF THE α - AND β -CHAINS OF HUMAN GLOBIN WITH ZINC IONS

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A number of findings [3, 8, 2] show that a prerequisite for better insight into the function of haemoglobin is the knowledge of mutual interactions of its subunits. Valuable information about these interactions can be gained from study of α - and β -chains even if, as comparison of haemoglobin and globin shows, the linkage to haem markedly alters their properties.

In the previous work [15] we studied the behaviour of α - and β -chains in globin at pH 7. By repeating several times slow neutralization of human acidglobin solutions we separated the non-aggregating and the aggregating portions, examined their chain compositions and molecular weights. The results show that α - and β -chains form complexes of various compositions and that the β -chain is more prone to aggregation.

The present study was undertaken with the objective of obtaining further information on the relative strength of linkages between the chains by studying the complexes of the individual chains and their mixtures with zinc ions.

EXPERIMENTAL

Materials. Human acidglobin was prepared by dropping slowly a 5% solution of haemoglobin into 15 vol. of acetone (-20°) containing HCl in a concentration of 0.015 N. The haemoglobin solution was obtained by diluting dialysed haemolysate of washed red cells. The stroma had been removed prior to dialysis by adsorption on an alumina gel, followed by filtration through a Seitz K pad.

Globin soluble in neutral medium was obtained by slow neutralization [15] of 3% acidglobin by dialysing against 0.05 N-sodium hydroxide solution to pH 7.

α - and β -chains of human globin were prepared according to Hayashi [6] by trichloroacetic acid precipitation in 8 M-urea. After dialysis and

freeze-drying the crude preparations were reprecipitated and freed from the bound trichloroacetic acid by dialysis at pH 9-10 and freeze-dried again. Their purity was estimated to be about 90% by paper electrophoresis according to Take [14] and by the method based on splitting off of the C-terminal amino acids [7].

Precipitation with zinc ions. A protein solution in water, buffer or urea was brought to pH 7 and mixed with equal volume of a ZnSO_4 solution of appropriate concentration. The given compositions of the systems (concentration of protein, urea and ZnSO_4 , and ionic strength) always refer to the final mixture. After 10 hr. of standing at room temperature the precipitate was centrifuged off and the amount of protein left in the supernatant was determined after dilution with 0.01 N-HCl, by measuring the extinction at 280 m μ in a Zeiss spectrophotometer.

Methods. The contents of the chains were estimated by electrophoresis at pH 6.3 according to Take [14] in a pyridine-acetate buffer which was 6 M in respect to urea. After 4 hr. of resolution at a potential gradient of 21 V/cm. and detection with bromophenol blue the chain contents were determined after elution [15] by photometry.

The sedimentation coefficients were measured in a Phywe ultracentrifuge at 48 000 r.p.m. and 18-20°. The values of $S_{20,w}$ were calculated by extrapolating the dependence of logarithms of the boundary positions upon the time of sedimentation and normalized to 20° and water as solvent. With the urea solutions the coefficients are uncorrected, because the presence of high concentrations of urea would require great corrections for which the formula currently employed (applicable in fact to bicomponent systems only) is not suitable [12].

RESULTS

The behaviour of separated chains. Either chain prepared by the trichloroacetic acid precipitation had a reduced solubility in the neutral pH region. The pH values of the minimum solubilities were 6.5 for the α -chain and 7.5 for the β -chain, in agreement with differences between their charges given by their amino acid compositions. At pH 5.37 and a ionic strength μ 0.03 (acetate buffer) both chains were well soluble. From the values of the sedimentation coefficients it follows that solutions of the β -chain contain mainly tetramers ($S_{20,w}=4.26$ S) whereas those of the α -chain are dimer-monomer mixtures ($S_{20,w}=2.84$ S).

Solubility of the chains is increased in the presence of urea. The α -chain is soluble even in 1 M-urea at pH 7. The β -chain proved well soluble in 3 M-urea at a ionic strength 0.04-0.4; i.e. that employed in the precipitation with zinc ions.

In the presence of zinc ions in 0.5–1% solutions of the β -chain in 3 M-urea, practically all the protein precipitates at 0.02 M concentration of zinc sulphate (Fig. 1). With a decrease in pH the amount of the precipitate is reduced; no precipitation occurs below pH 5. The amount of the precipitable protein also decreases with increasing concentration of urea (Fig. 2). The α -chain, on the other hand, did not precipitate at all.

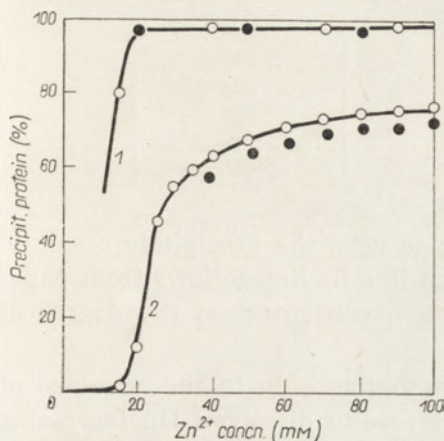


Fig. 1

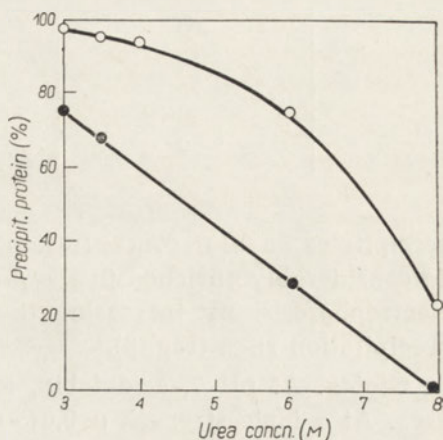


Fig. 2

Fig. 1. Amount of the precipitated protein in relation to molar concentration of zinc ions. 1, (○), 0.5% and (●), 1% solutions of the β -chain in 3 M-urea. 2, (○), 0.5% solution of a mixture composed of 50% α - and 50% β -chains in 3 M-urea and (●), 0.5% solution of the soluble globin fraction in 0.05 M-NaCl.

Fig. 2. Amount of the precipitated protein in relation to molar concentration of urea. 0.5% protein solutions, 0.1 M- Zn^{2+} ; (○), the β -chain; (●), equimolar mixture of α - and β -chains.

A mixture of 50% α - and 50% β -chains. On mixing solutions of the chains in acetate, pH 5.37, μ 0.03, a precipitate is formed. If, however, one of the two chains is present in excess the precipitate dissolves after a certain time. As can be seen from Table 1 the sedimentation analysis exhibits only one boundary, whose sedimentation coefficient gradually rises with the increasing content of the β -chain. On mixing equimolar amounts of the chains practically all the protein irreversibly precipitates. The sedimentation coefficient of a 0.8% solution of this precipitate in 8 M-urea at pH 7 was 1.20 S, the same as that of the β -chain solution of equal concentration and in the same medium (1.22 S). At 0.1 M concentration of zinc ions about 75% of the protein precipitates from a 0.5% solution of the equimolar mixture of chains in 3 M-urea (Fig. 1). With an increase in the concentration of urea the amount of the precipitable portion decreases (Fig. 2). In 5 M-urea, where 45% of the material

Table 1

*Sedimentation coefficients of mixtures of α - and β -chains*Acetate buffer pH 5.37, μ 0.03, protein concentration 0.8%.

α -Chain (%)	β -Chain (%)	$S_{20,W} \times 10^{13}$
100	0	2.84
80	20	2.93
35	65	3.24
20	80	3.86
0	100	4.26

precipitates at 0.1 M concentration of zinc ions, the precipitate seems to be considerably enriched in the β -chain; this finding follows from paper electrophoresis. By increasing the protein concentration the degree of precipitation rises (Fig. 3).

Globin. At pH 7, acidglobin, too, is soluble only in the presence of urea. At a ionic strength μ 0.04 - 0.6 it readily dissolves. On increasing the ionic strength to a value of 0.6 to 1.6 it is salted out. In a fresh 3 M-solution of urea no precipitation occurs in the presence of zinc ions. If, however, the urea solution was about 1 month old, or had been

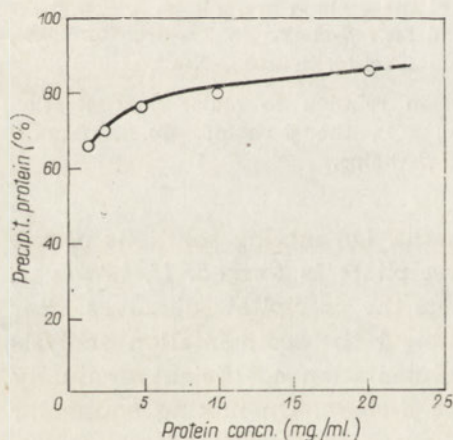


Fig. 3. Effect of protein concentration on the amount of precipitate. 0.5% solution of equimolar mixture of α - and β -chains in 3 M-urea at 0.1 M- Zn^{2+} .

heated at 80° for half-an-hour, the precipitation took place. For a 0.5% acidglobin solution the curve of precipitation with zinc ions is practically identical with that for an artificial mixture of the chains (Fig. 1). However, the precipitation of globin is much slower and at higher protein concentrations less precipitate is formed than with the mixture of chains, because the amount of zinc ions necessary for the precipitation

is a linear function of the protein concentration in a range from 0.2 to 1%.

We have failed to explain the effect of age of the urea solution observed with globin. By measuring the sedimentation coefficients we found that the degree of aggregation of globin molecules is the same in both a fresh and an old solution of urea. The sedimentation coefficients uncorrected for the medium of 3 M-urea solution were 2.78 S and 2.80 S, respectively.

From 0.5% solution of globin prepared by dialysing acidglobin against 0.05 N-NaOH to pH 7 about 70% of the protein precipitates at zinc ion concentration 0.05 - 0.1 M in the absence of urea. The precipitation curve is again practically identical with that of the artificial mixture of the chains in 3 M-urea. In the presence of urea the soluble fraction of globin behaves as the starting acidglobin.

DISCUSSION

The accumulation of β -chains in portions of human globin insoluble at pH 7, observed in the previous work [15], and the dependence of sedimentation coefficients of these fractions upon pH, suggest higher aggregability of the β -chain, compared to the α -chain. The values of sedimentation coefficients at pH 5.37 show that even when isolated, the β -chain forms higher aggregates and, as a consequence, its solubility faster decreases with a decrease in the net charge. It is gradually less soluble than the α -chain both in buffers and in urea. Also with horse globin the molecular weight of the β -chain at pH 1 - 5 was higher than that of the α -chain [5, 16]. The behaviour of native haemoglobin chains also testifies to a higher aggregability of the β -chain. Whereas haemoglobin H, composed solely of β -chains, has a molecular weight corresponding to the tetramer β_4 [11], the molecular weight of haemoglobin α , isolated from the haemoglobin of adults by chromatography on carboxymethylcellulose, was 25 030 to 32 100; this value corresponds to a mixture of 40% of a dimer and 60% of the monomer [13].

The difference in aggregability of α - and β -chains is in accordance with that in solubility of their complexes with zinc ions. Complexes of the β -chain with zinc ions in 3 to 4 M-urea at pH 7 are quite insoluble whereas solutions of α -chains do not precipitate with zinc ions under these conditions. It seems that this fact might be made use of for separation of the two types of chain. In mixtures, however, the linkages between the α - and β -chains obviously lead to the formation of complexes non-cleavable in 3 M-urea so that, depending on the conditions, either a part of the α -chains co-precipitates with the β -chains or, conversely, part of the β -chains remains in solution with the α -chains. For if we assumed that only β -chains, which will be in equilibrium with the

complexes, are precipitable, the amount of the precipitate could not exceed the total quantity of β -chains; this, however, is at variance with experiment.

The strong mutual affinity of α - and β -chains is also suggested by their co-precipitability at pH 5.37. If the two chains are mixed in an equimolar ratio the aggregates are quite insoluble whereas if one chain is present in excess the complexes are soluble. This behaviour resembles the system antigen - precipitating antibody.

The fact that even several slow precipitations of acidglobin fractions differing in solubility at pH 7 result in only poor enrichment in the individual chains is evidence for the existence of strong α - β linkages. Alternatively, of course, owing to the great difference in aggregability, the separation of the chains would have to be practically complete. Great mutual affinity of the two chains has also been observed in their native states. Haemoglobin α and haemoglobin H immediately form haemoglobin A, even without previous acidification [10, 9].

Hybridization experiments have so far been generally interpreted in the sense of asymmetrical cleavage of the haemoglobin molecule. However, there have progressively been observed a number of facts [see 1] that militate against this view. If the behaviour of separated chains of globin allows to draw conclusions on their behaviour in haemoglobin it proves that there is a strong mutual affinity between the two types of chain and thus substantiates the view that the cleavage of the haemoglobin molecule is symmetrical.

Interesting differences can be observed between the behaviour of globin and that of artificial equimolar mixtures of α - and β -chains in precipitation with zinc ions in the presence of urea. These differences can probably be ascribed to the upset of conformations of the chains caused by their separation.

In view of the great specificity of the α - β bonds shown in some hybridization experiments with haemoglobins from various animals [1], it can be expected that the nature of the binding will be similar to that in antigen-antibody systems. The binding is probably mediated by sterically complementary regions with the participation of non-covalent linkages. The model [4] of the spatial structure in crystalline state has afforded no reliable information on the nature of these linkages. It only seems that the α - α linkages are participated in mainly by groups in helix H (probably Lys¹²⁷, Ser¹³³ and Ser¹³⁸, Thr¹³⁴ and Thr¹³⁷) and the β - β linkages by groups in helix G (probably Arg¹⁰⁴ and Asp¹⁰⁶). Another possibility, acceptable with both types of chain, is the linkage of the N-terminal α -amino groups to the C-terminal carboxyls. It is natural that the removal of haem from the molecule probably deeply alters the conformations of the chains, so that the linkages occurring in the isolated

globin chains may not be identical with those in the haemoglobin molecule. To obtain more accurate information about their nature it will, of course, be necessary to study the behaviour of chain mixtures under most diverse conditions.

SUMMARY

Differences in solubility and sedimentation coefficients of the isolated chains confirm the easier aggregability of β -chains, observed in the previous study of human globin fractions differing in solubility at pH 7.

When in the isolated form, in the presence of 3 to 5 M-urea at pH 6 - 7, only the β -chain forms insoluble complexes with zinc ions. In mixtures of chains, however, their mutual linkages considerably modify their behaviour toward zinc ions. A strong mutual affinity of the two types of chain can also be deduced from their co-precipitation. At equimolar ratios the complexes are quite insoluble at pH 5.37, but they dissolve when one of the two chains is present in excess. The behaviour of mixtures of separated chains favours the hypothesis about the symmetrical cleavage of the haemoglobin molecule.

Marked differences between the behaviour of globin and that of experimentally prepared mixtures of 50% α - and 50% β -chains, in the presence of urea and zinc ions, clearly prove the conformations of the isolated chains to be different from those in the starting globin.

REFERENCES

- [1] Antonini E., Wyman J., Bucci E., Fronticelli C. & Rossi-Fanelli A. - *J. Mol. Biol.* **4**, 368, 1962.
- [2] Benesch R. & Benesch R. E. - *J. Mol. Biol.* **6**, 498, 1963.
- [3] Benesch R. E., Ranney H. M., Benesch R. & Smith M. - *J. Biol. Chem.* **236**, 2926, 1961.
- [4] Cullis A. F., Muirhead H., Perutz M. F., Rossmann M. G. & North A. C. T. - *Proc. Roy. Soc. A* **265**, 161, 1962.
- [5] Haugh A. & Smith D. B. - *Canad. J. Chem.* **35**, 945, 1957.
- [6] Hayashi H. - *J. Biochem. (Tokyo)* **50**, 70, 1961.
- [7] Holeyšovská H., Holeyšovský V. & Vodrážka Z. - *Collection Czechoslovak Chem. Comm.* **29**, 1282, 1964.
- [8] Horton B. F., Thompson R. B., Dozy A. M., Nechtman C. M., Nicols E. & Huisman T. H. J. - *Blood* **20**, 302, 1962.
- [9] Huehns E. R. & Beaven G. H. - *Biochem. J.* **83**, 40P, 1962.
- [10] Huehns E. R. & Shooter E. M. - *Nature* **193**, 1083, 1962.
- [11] Jones R. T. W., Schroeder W. A., Balog J. E. & Vinograd J. R. - *J. Am. Chem. Soc.* **81**, 3161, 1959.
- [12] Schachman H. K., *Ultracentrifugation in Biochemistry*, p. 82, Academic Press, New York 1959.
- [13] Shooter E. M., *Internationales Symposium über Molekulare Zellphysiologie, Berlin 1963*. Springer Verlag, 1964.

- [14] Take T. - *J. Biochem. (Tokyo)* **49**, 206, 1961.
[15] Vodrážka Z., Holeyšová H. & Šípalová H. - *Collection Czechoslovak Chem. Comm.* **29**, 1287, 1964.
[16] Wilson S. & Smith D. B. - *Canad. J. Biochem. Physiol.* **37**, 405, 1959.

INTERAKCJA ŁAŃCUCHÓW α i β GLOBINY LUDZKIEJ Z JONAMI CYNKU

Streszczenie

Stwierdzono różnice w rozpuszczalności i współczynniku sedymentacji między izolowanymi łańcuchami α i β . Potwierdza to poprzednie obserwacje o łatwiejszym tworzeniu agregatów przez łańcuchy β .

W obecności 3 - 5 M-mocznika w pH 6 - 7 tylko łańcuch β tworzy nierozpuszczalne połączenia z jonami cynku. W mieszaninie wzajemne połączenia łańcuchów znacznie zmieniają ich zachowanie wobec jonów cynku. Dowodem na wzajemne powinowactwo łańcuchów jest również ich ko-precypitacja. Kompleksy równomolarne są nierozpuszczalne w pH 5.37; rozpuszczają się po dodaniu jednego z łańcuchów w nadmiarze. Zachowanie się mieszanin wyizolowanych łańcuchów wskazuje na słuszność hipotezy o symetrycznym rozkładzie cząsteczki hemoglobiny.

Znaczne różnice w zachowaniu globiny oraz mieszaniny łańcuchów 50% α i 50% β w obecności mocznika i jonów cynku dowodzą, że struktura izolowanych łańcuchów jest różna od struktury wyjściowej globiny.

Received 3 February 1964.

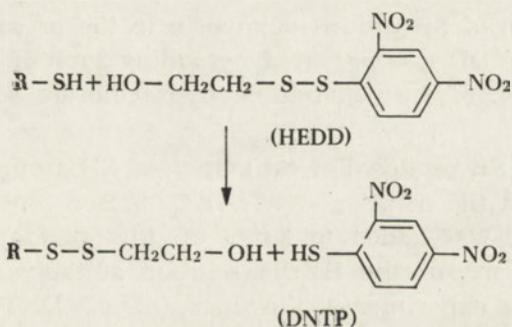
IRENA KĄKOL, J. GRUDA and S. BITNY-SZLACHTO*

A STUDY ON THE ROLE OF SH GROUPS OF MYOSIN BY MEANS OF β -HYDROXYETHYL-2,4-DINITROPHENYL DISULPHIDE

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The role of SH groups in myosin and in other muscle proteins has been intensively investigated during the last years by many authors but many questions still remain unsolved.

In the present paper β -hydroxyethyl-2,4-dinitrophenyl disulphide (HEDD¹) was used in an attempt to characterize the SH groups of myosin. HEDD reacts with the SH groups of different compounds forming corresponding disulphides and yellow coloured DNTP [2, 3] (Scheme 1).



Scheme 1

The course of the reaction can be followed by measuring the extinction at 408 m μ . The increase of the extinction of the mixture corresponds to the amount of DNTP formed and to the amount of thiol groups participating in the reaction. The use of HEDD permits in this way to follow the blocking of SH groups and to study the changes of the properties of the protein².

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¹ Abbreviations used: HEDD, β -hydroxyethyl-2,4-dinitrophenyl disulphide; DNTP, 2,4-dinitrothiophenol.

² Some of the results were presented at the 1st Polish Congress of Biochemistry, Łódź, September 1963.

MATERIALS AND METHODS

Preparation of proteins and chemicals used. Myosin was obtained from skeletal rabbit muscles by 10 min. extraction with the Edsall-Weber solution and purified according to Portzehl & Weber [18]. The obtained solution of myosin in 0.5 M-KCl was cleared up by centrifugation for 2 hr. in a Spinco ultracentrifuge (Rotor 40.2) at 109 000 g.

Actin was extracted at 0° [7] from acetone-dried rabbit muscles according to Feuer *et al.* [9] and purified according to Mommaerts [17].

HEDD was obtained according to Böhme & Stachel [6], and DNTP according to Wilgerodt [22]. Nucleotides were Pabst (U.S.A.) products. Other chemicals were reagent grade. Water used in the course of this work was deionized on Elgastat deionizer.

Preparation of HEDD solution. The 0.5 mM standard solution of HEDD was freshly prepared before each experiment in the following way: 2 ml. of 0.025 M-HEDD solution in ethanol were added to 80 ml. of the buffer solution (0.025 M-tris - acetate buffer, pH 6.8, containing 0.5 M-KCl) at 40°; the volume was adjusted to 100 ml. with the same buffer solution and the 0.5 mM-HEDD obtained was filtered through VEB 388 h filter paper.

For estimation of SH groups of myosin in the presence of guanidine the solution of HEDD was prepared by adding 2 ml. of 0.025 M-HEDD in ethanol to 100 ml. of 2.5 M-guanidine hydrochloride solution in water, pH 6.8.

Estimation of SH groups. For estimation of SH groups 1 ml. of a solution of myosin in the mentioned tris - KCl buffer containing from 2 to 4 mg. protein/ml. was added to 3 ml. of the standard 0.5 mM-HEDD solution or to 3 ml. of the HEDD solution suitably diluted with the buffer. In all the experiments the increase of DNTP concentration, resulting from the interaction of the myosin SH groups with HEDD, was determined in the Unicam 600 spectrophotometer in 1 cm. cells at 408 m μ against blanks containing appropriate solutions of HEDD.

The value 13 060 was accepted as the molar extinction E_{408} of DNTP [3] and 12 600, respectively, when the estimations were performed in the presence of 2.5 M-guanidine. The extinction at 408 m μ of the myosin solution without HEDD was checked every time; at the concentrations used it was practically negligible (less than 0.005).

Estimation of enzymic activity of myosin. In the studies on the effect of blocking SH groups on the enzymic activity of myosin 30 mg. of protein dissolved in 5 ml. of buffer solution were preincubated for 2 hr. at room temp. with 25 ml. of buffer solution containing up to 40 moles of HEDD per 10⁵ g. of myosin. The amount of SH groups having reacted

during this time was estimated as above. Throughout the work the amount of Eq. SH has been calculated per 10^5 g. myosin.

For determination of ATPase activity, to each 1 ml. sample of the mentioned mixtures 1 ml. of the buffer solution was added containing 10 μ moles of ATP and 10 μ moles of Ca^{2+} or EDTA. After 5 min. incubation at 25° the reaction was stopped by adding 2 ml. of 20% TCA. The orthophosphate released was determined according to Fiske & Subbarow [10]; it was shown in a control test that neither HEDD nor DNTP interfered with the estimation of orthophosphate by this method.

The ATPase activity of myosin treated with HEDD was expressed as percentage of the activity of the control myosin determined in the presence of Ca^{2+} .

Estimation of actomyosin formation ability. For studying the effect of blocking SH groups on the ability of myosin to bind actin, 2 ml. samples of myosin solution (10 mg./ml.) were preincubated for 24 hr. at 3° with 8 ml. of buffer solution containing HEDD. The amount of HEDD varied from 0 to 10 moles per 10^5 g. of myosin. Within 24 hr. the reaction of a definite amount of SH groups, dependent on the concentration of HEDD, was practically completed. After warming the mixture to room temperature the amount of bound SH groups was estimated. Next, to a sample of the mixture a solution of F-actin was added in an amount which corresponded to the weight ratio of F-actin to myosin as 1:4. After 1 hr. the viscosity of actomyosin formed was measured with Oswald viscometer at 22° . The ability of myosin preincubated with HEDD to form a complex with actin was expressed as percentage of the activity of the native myosin according to Bárány & Bárány [1]. It was calculated from the equation:

$$\text{Percentage activity} = \frac{\log \eta_{\text{rel}}(A + M_{\text{HEDD}}) - (\log \eta_{\text{rel}} M_{\text{HEDD}} + \log \eta_{\text{rel}} A)}{\log \eta_{\text{rel}}(A + M) - (\log \eta_{\text{rel}} M + \log \eta_{\text{rel}} A)} \times 100.$$

Where $\eta_{\text{rel}}(A + M_{\text{HEDD}})$ is the viscosity of actomyosin solution prepared from myosin samples treated with HEDD; $\eta_{\text{rel}}(A + M)$, relative viscosity of actomyosin solution prepared from myosin samples without HEDD; $\eta_{\text{rel}} M_{\text{HEDD}}$, relative viscosity of myosin solution treated with HEDD; $\eta_{\text{rel}} M$, relative viscosity of myosin solution without HEDD; $\eta_{\text{rel}} A$, relative viscosity of F-actin solution.

In all the experiments myosin was added to actin without a previous removal of HEDD and of the formed DNTP. Such a procedure was based on our as well as on Drabikowski's & Bitny-Szlachto's [8] observations which showed that these two substances did not affect the ability of actin to form a complex with myosin.

Protein was determined with the use of biuret reagent [12],

standardized on the basis of the estimation of protein by the Kjeldahl method; the nitrogen content in the protein being accepted as 16.7%.

The pH of the solutions was estimated on a Radiometer PHM-22 with accuracy ± 0.02 .

RESULTS

The time course of the reaction of SH groups of myosin with varying concentrations of HEDD is shown in Fig. 1. The curve *E* presents the results of experiments in which the amount of HEDD corresponds to a 7-fold excess of the reagent in respect to the accepted number of

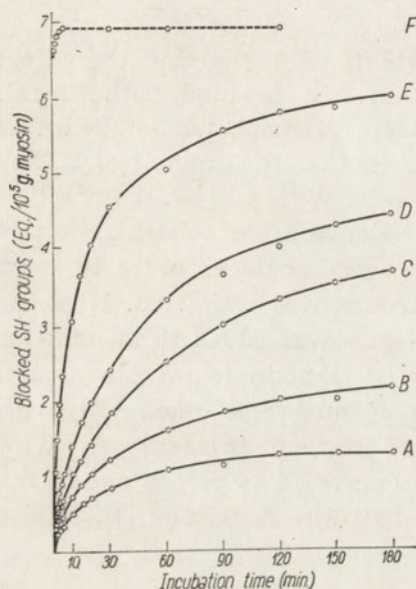


Fig. 1. Reaction between myosin and HEDD. Each sample contained 2.8 mg. of myosin. Total volume 4 ml., temp. 20°. The samples from *A* to *E* contained: 0.5 M-KCl; 0.025 M-tris-acetate buffer, pH 6.8, and the following amounts of HEDD expressed in moles per 10^5 g. myosin: *A*, 1.4; *B*, 2.85; *C*, 5.9; *D*, 10.5; *E*, 52.5. The sample *F* contained 2.5 M-guanidine-HCl, pH 6.8, and HEDD 52.5 moles/ 10^5 g. myosin.

7.5 Eq. SH per 10^5 g. myosin. It can be seen that already after 3 min. the extinction value found at 408 m μ corresponded to the blocking of 2 Eq. SH. Subsequent 2 Eq. SH were found to be attacked by HEDD towards the next 20 min. and during 2 hr. of incubation 6 Eq. SH in total appeared to react with HEDD.

The increase of the concentration of HEDD to a 14-fold excess did not change the course of the reaction. On the other hand the decrease of the excess to a 1.5-fold (curve *D*) diminished the rate of the reaction. Thus it was found that in such case only about 4 Eq. SH of myosin reacted during 2 hr. incubation. The curves *A*, *B* and *C* in the same figure show the time course of the reaction with amounts of HEDD deficient in respect to the amounts of SH groups. In all these cases a more pronounced decrease of the reaction rate was observed and, besides, a part of HEDD failed to react with myosin.

Denaturing agents caused both a considerable acceleration of the reaction rate and an increase of the total amount of SH groups reacting with HEDD (curve F). Thus, in the presence of 2.5 M-guanidine hydrochloride the extinction corresponding to about 7 Eq. SH was observed already after a few minutes. The disorganization of the secondary structure of the protein molecule which evidently takes place under influence of guanidine enables almost all SH groups of myosin to react with HEDD during a very short period of time. The value of about 7 Eq. SH per 10^5 g. of myosin obtained in the presence of guanidine hydrochloride closely corresponded to the results of amperometric determinations of Kominz *et al.* [16] and Szent-Györgyi [21].

To compare the reaction rate of HEDD with myosin with that of other compounds containing SH groups similar experiments were performed with glutathione. The excess of the reagent, pH, and ionic strength were the same as described above. As can be seen (Fig. 2) the

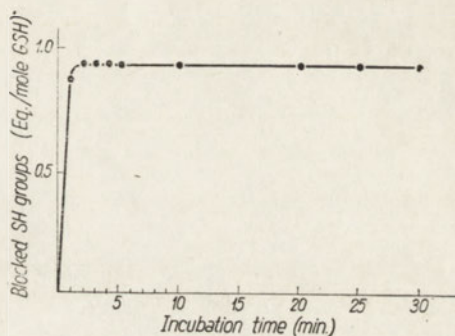


Fig. 2. Reaction between glutathione and HEDD. The sample contained: 0.5 M-KCl; 0.025 M-tris - acetate buffer, pH 6.8; glutathione, 61.4 μ g. (0.2 μ mole); and HEDD, 1.5 μ moles. Total volume 4 ml., temp. 20°.

reaction was practically finished during 2 min. and the values obtained for the amount of SH groups were close to the theoretical ones.

The reaction of myosin SH groups with HEDD was not appreciably affected by addition of ATP, ITP, ADP, AMP, PP or EDTA at final concentration of 10^{-3} M. It was observed that the rate of the reaction of SH groups of native myosin with HEDD depended on pH of the incubation mixture. Fig. 3 shows that even a small decrease of pH delayed the rate of the reaction. For that reason in the experiments with ATP and ITP final concentration of tris - acetate buffer was increased to 0.1 M in order to avoid the decrease of pH occurring during the enzymic hydrolysis of the nucleotides.

The blocking of SH groups by HEDD had a distinct effect on the ATPase activity of myosin (Fig. 4). When the protein was treated with small amounts of HEDD, and myosin ATPase was assayed in the presence of Ca^{2+} , a considerable increase of enzymic activity was noted. The maximum of activation was observed when 1.5 Eq. SH had been blocked. The blocking of a larger amount of SH groups decreased and,

finally, stopped the enzymic activity. On the other hand, no activation was observed when instead of Ca^{2+} EDTA was added. In this case a complete inhibition of the enzymic activity occurred already when 2 SH groups of myosin were blocked.

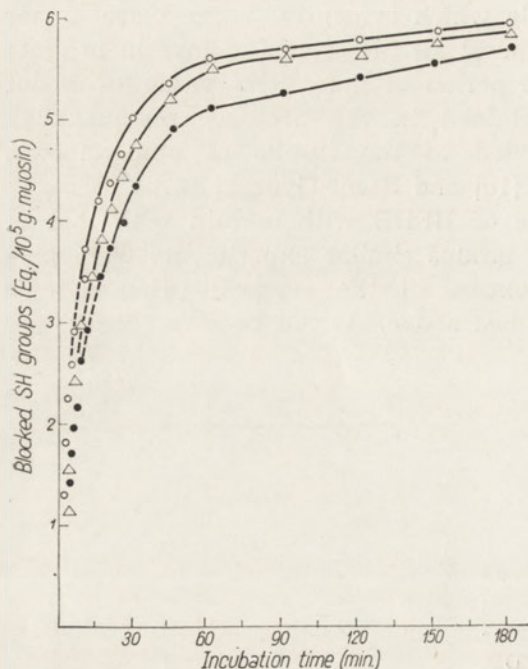
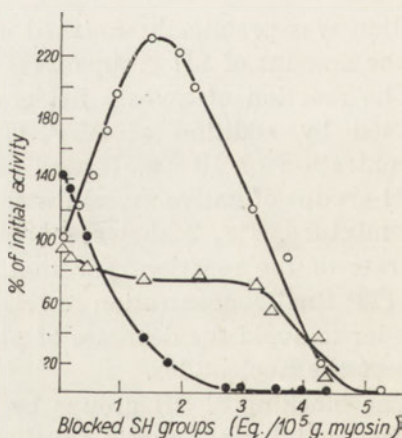


Fig. 3. Effect of pH on the reaction between myosin and HEDD. Content of the sample: 2.2 mg. myosin; HEDD, 34 moles/ 10^5 g. myosin; 0.5 M-KCl; 0.025 M-tris-acetate buffer of various pH. Total volume 4 ml., temp. 20°. (●), pH 6.7; (Δ), pH 6.8; (○), pH 6.9.

Fig. 4. Effect of blocking of SH groups of myosin with HEDD on its ATPase activity and on its ability to form actomyosin. (○), ATPase activity in the presence of Ca^{2+} ; (●), ATPase activity in the presence of EDTA; (Δ), actomyosin formation activity (see Methods).



As it was proved in control experiments DNTP, at concentrations formed during the reaction of myosin with HEDD, did not affect the ATPase activity of myosin.

The effect of blocking varying number of SH groups on the ability of myosin to form a complex with actin is shown in Fig. 4. The blocking of 3 Eq. SH of myosin somewhat decreased the formation of actomyosin, while after blocking of a larger number of SH groups the decrease was very conspicuous.

DISCUSSION

The results of experiments presented above agree with the hypothesis of heterogeneity of the SH groups in myosin. The data obtained indicate that only a part of the SH groups reacts rapidly with HEDD. The rate of the reaction of these groups was similar to that of cysteine [3] or glutathione as well as that of the easily available SH groups of some proteins, as e.g. all SH groups in bovine serum albumin [3] or a part of SH groups in the native actin molecule [8]. Another part of SH groups of myosin reacts considerably slower, so that the reaction lasts several hours. It can be supposed that this kind of SH groups is partly "masked" by the secondary structure of the protein. A third kind of SH groups of myosin seems to be not accessible for the reagent used and reacts only after disorganization of the secondary structure as in the case of treatment with guanidine or after a long incubation of the protein solution at room temperature.

Comparing the changes of ATPase activity of myosin with the number of the blocked sulphhydryl groups it can be concluded that the most reactive towards HEDD are those groups whose blocking causes an increase of ATPase activity in the presence of Ca^{2+} and an inhibition in the presence of EDTA. Thus, HEDD has appeared to be a reagent acting similarly to NEM and PCMB which are commonly used in this type of studies. NEM and PCMB after blocking of 1 to 2 Eq. SH, also activate ATPase in the presence of Ca^{2+} and only after blocking of a larger number of SH groups a gradual decrease of the enzymic activity may be observed [14, 11, 20, 5]. It is to be emphasized that this biphasic response of myosin ATPase takes place only in the presence of Ca^{2+} ; also in this respect the effect of HEDD is similar to that of NEM and PCMB [14, 4, 11]. In the presence of EDTA a complete inactivation of ATPase by HEDD is observed already after blocking of 2 SH groups.

In recent studies, using ^{14}C -labelled NEM [19, 13], additional evidence was obtained for the existence of some differences in the reactivity of particular SH groups of myosin involved in the enzymic process. It can be supposed therefore that at least two kinds of SH groups are in a different way involved in the reaction of ATP hydrolysis. Besides this, it seems that myosin contains also an other kind of SH groups which is responsible for the formation of the complex with actin. This was demonstrated by Bárány & Bárány [1] who by treating actomyosin with iodo-

acetamide obtained a preparation of myosin deprived of ATPase activity but still retaining the complex-forming ability. The present experiments also indicate that only some of the SH groups are necessary for the reaction with actin and that these groups do not react rapidly with HEDD. This is in accordance with the fact that the formation of the actomyosin complex is inhibited only after blocking of more than 3 Eq. SH per 10^5 g. of myosin.

We should like to thank Professor W. Niemierko for his interest and advice. We are also grateful to Mrs. T. Kośmicka for her skilled assistance throughout the course of the work.

SUMMARY

1. A study was made on the influence of blocking various number of SH groups of myosin by β -hydroxyethyl-2,4-dinitrophenyl disulphide on the ATPase activity of this protein and its ability to bind with actin.

2. In the presence of a large excess of the reagent during 2 hr. about 6 SH Eq./ 10^5 g. of native myosin were gradually blocked. After denaturation of myosin with guanidine practically all SH groups, i.e. about 7 Eq./ 10^5 g. were blocked already in a few minutes.

3. Blocking of only a part of SH groups activated the ATPase activity of myosin in the presence of Ca^{2+} . Maximum activation was found after blocking of about 1.5 Eq./ 10^5 g. However, blocking of a larger quantity of SH groups diminished or even completely inhibited the enzymic activity. If after blocking of a part of SH groups ATPase was estimated in presence of EDTA instead of Ca^{2+} , no increase but only inhibition of the enzymic activity was found.

4. Blocking of more than 3 SH Eq./ 10^5 g. of myosin completely abolished the ability to combine with actin, while blocking of a smaller amount of SH groups diminished this ability only inconsiderably.

5. On ground of these results the role of particular SH groups in myosin is discussed.

REFERENCES

- [1] Bárány M. & Bárány K. - *Biochim. Biophys. Acta* **35**, 293, 1959.
- [2] Bitny-Szlachto S. - *Acta Polon. Pharm.* **17**, 373, 1960.
- [3] Bitny-Szlachto S., Kosiński J. & Niedzielska M. - *Acta Polon. Pharm.* **20**, 365, 1963.
- [4] Blum J. J. - *Arch. Biochem. Biophys.* **87**, 104, 1960.
- [5] Blum J. J. - *Arch. Biochem. Biophys.* **97**, 309, 1962.
- [6] Böhme H. & Stachel M. D. - *Z. Anal. Chem.* **154**, 27, 1957.
- [7] Drabikowski W. & Gergely J. - *J. Biol. Chem.* **237**, 3412, 1962.
- [8] Drabikowski W. & Bitny-Szlachto S. - *Bull. Acad. Polon. Sci., Ser. Biol.* **11**, 165, 1963.

- [9] Feuer G., Molnar F., Pettko E. & Straub F. B. - *Hung. Acta Physiol.* **1**, 150, 1948.
- [10] Fiske C. H. & Subbarow Y. - *J. Biol. Chem.* **66**, 375, 1925.
- [11] Gilmour D. & Gellert M. - *Arch. Biochem. Biophys.* **93**, 605, 1961.
- [12] Gornall A. G., Bardawill Ch. J. & David M. M. - *J. Biol. Chem.* **177**, 751, 1949.
- [13] Gröschel-Stewart U. & Turba F. - *Biochem. Z.* **337**, 104, 1963.
- [14] Kielley W. W. & Bradley L. B. - *J. Biol. Chem.* **218**, 653, 1956.
- [15] Kitagawa S., Yoshimura I. & Tonomura Y. - *J. Biol. Chem.* **236**, 902, 1961.
- [16] Kominz D. R., Hough A., Symonds P. & Laki K. - *Arch. Biochem. Biophys.* **50**, 148, 1954.
- [17] Mommaerts W. F. H. M. - *J. Biol. Chem.* **198**, 469, 1952.
- [18] Portzehl H., Schramm G. & Weber H. H. - *Z. Naturforsch.* **5b**, 61, 1950.
- [19] Sekine T., Barnett L. M. & Kielley W. W. - *J. Biol. Chem.* **237**, 2769, 1962.
- [20] Stracher A. & Chan P. C. - *Arch. Biochem. Biophys.* **95**, 435, 1961.
- [21] Szent-Györgyi A. G., Benesch R. E. & Benesch R., in *Sulfur in Proteins* (Benesch R., Benesch R. E., Boyer P. D., Klotz J. M., Middlebrook W. R., Szent-Györgyi A. G. & Schwarz D. R., eds.) p. 291, Academic Press, New York, 1959.
- [22] Wilgerodt C. - *Ber. Deutsch. Chem. Ges.* **17**, Ref. 352, 1884.

BADANIE ROLI GRUP SH MIOZYNY PRZY POMOCY DWUSIARCZKU β -HYDROKSYETYLO-2,4-DWUNITROFENYLOWEGO

Streszczenie

1. Badano wpływ blokowania różnej ilości grup SH miozyny przy pomocy dwusiarczku β -hydroksyetylo-2,4-dwunitrofenylowego na ATPazową aktywność tego białka i na jego zdolność łączenia się z aktyną.

2. W obecności znacznego nadmiaru odczynnika w ciągu dwóch godzin ulegało stopniowemu zablokowaniu 6 równoważników SH/10⁵ g. natywnej miozyny. Po zdenaturowaniu zaś miozyny chlorowodorkiem guanidyny użyty dwusiarczek już w ciągu kilku minut blokował praktycznie biorąc wszystkie grupy SH tego białka, tj. około 7 równoważników na 10⁵ g. miozyny.

3. Zablokowanie tylko pewnej części grup SH aktywowało, w obecności Ca²⁺, ATPazę miozynową. Maksimum tej aktywności obserwowano po zablokowaniu około dwóch równow. SH, natomiast zablokowanie większej ilości grup SH prowadziło do zmniejszenia aktywności enzymatycznej aż do jej całkowitego zaniku. Stosując EDTA zamiast Ca²⁺ stwierdzono po zablokowaniu grup SH jedynie zahamowanie aktywności ATPazowej.

4. Zablokowanie ponad 3 równow. SH miozyny powodowało całkowitą utratę zdolności do łączenia się tego białka z aktyną, zablokowanie zaś mniejszej ilości grup SH jedynie nieznacznie wpływało na to zjawisko.

5. Na podstawie uzyskanych wyników przeprowadzono dyskusję nad rolą poszczególnych grup SH w cząsteczce miozyny.

Received 28 December 1963.

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STUDIES ON SULPHYDRYL GROUPS OF ACTIN

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In the recent years problems relating to the sulphydryl groups in actin have become an important topic in muscle biochemistry. One aspect of this problem concerns the number of SH groups present in actin molecule. Several workers [22, 14, 23, 24] using various methods of determination showed recently that actin molecule contains 6 to 7 SH groups. The second important aspect in this field concerns the role of sulphydryl groups in actin molecule. It was earlier shown that mercurials inhibit polymerization of G-actin [16] and also cause the release of bound nucleotide [2, 19, 23]. Recently Drabikowski & Gergely [8] using various reagents were able to separate these processes and to show that actin molecule contains at least three kinds of SH groups: one kind, which apparently is not connected with any known specific property of actin, directly reacts with *N*-ethylmaleimide (NEM) [25]; the second kind is involved in polymerization of this protein, and the third is more or less directly connected with the nucleotide binding.

Since NEM, contrary to mercurials, combines only with a part of SH groups in native actin molecule [25, 2, 14, 23, 24] it seemed of interest to examine from this point of view disulphides, another class of compounds reacting with the protein SH groups. As an example β -hydroxyethyl-2,4-dinitrophenyl disulphide (HEDD) has been chosen. This reagent appears to be suitable for this purpose since as a result of its reaction with SH groups a stoichiometrical liberation of dinitrothiophenol takes place and this latter compound can be easily determined spectrophotometrically [3, 4]. In the present paper the number of directly reacting SH groups as well as the total number of SH groups after denaturation of actin have been determined using HEDD. The influence of this reagent on specific properties of actin has been also investigated. A preliminary report of the results presented here appeared earlier [9].

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MATERIAL AND METHODS

Purified G-actin was prepared as described previously [7]. Heat denaturation of actin was carried out by incubation of G-actin for 10 min. in a boiling water bath.

Actin containing bound [^{14}C]ATP was prepared by incubation of G-actin with [^{14}C]ATP for 20 min. at room temp. [18]. When necessary, free nucleotides were removed from actin solutions by treatment for 3 min. with Dowex 1 (Cl^- form) by adding the resin suspension in 2 mM-tris - HCl, pH 8.0, to a final Dowex concentration of 0.08 to 0.1 mEq. per ml. of actin. Radioactivity of the samples was measured using a Chicago Nuclear Corporation gas-flow counter with "Micromil" window.

HEDD was prepared according to Böhme & Stachel [5]. Stock solutions of 0.025 M-HEDD in 96% ethanol were diluted 50-fold with 2 mM-tris - HCl, pH 7.0, directly before use. The reaction of actin with HEDD was performed at room temperature. In the routine assays the final concentration of actin was 1.0 to 1.5 mg. per ml. and that of HEDD was 0.3 to 0.4 mM. The amounts of dinitrothiophenol formed during the reaction were measured in a Unicam Spectrophotometer SP 600 at 408 m μ , using 13060 as a molar extinction coefficient for dinitrothiophenol [4].

Protein concentration was determined with a biuret reagent [10a]. Viscosity measurements were carried out in Ostwald viscometers with outflow time of approximately 50 sec. at 21°.

NEM was purchased from Sigma Chemical Company, USA. Crystalline ATP, disodium salt (Pabst Laboratories, USA) and [^{14}C]ATP, disodium salt (Schwarz BioResearch, Inc., USA) were obtained as a generous gift from the Rockefeller Foundation.

RESULTS

The time course of the reaction of G-actin with HEDD, both in the absence and in the presence of various concentrations of EDTA, is presented in Fig. 1. It can be seen that in native G-actin only two equivalents of sulphhydryl groups per 60 000 g. (the value accepted as the molecular weight of G-actin) react with HEDD. The obtained values varied in particular experiments from 1.7 to 2.2 SH Eq. per mole of actin. In the conditions of our experiments, with a 20-fold excess of HEDD over the molar concentration of actin, the reaction proceeds very fast and is finished within 5 to 10 min. If actin preparations contain 0.2 mM free ATP a further incubation with HEDD is practically without any effect. Only if actin had been previously deprived of free ATP by treatment with Dowex 1, as in the experiment presented in Fig. 1, a very slow reaction with HEDD, proceeding further, can be observed; this is probably due to a slow denaturation of G-actin in the absence of free ATP.

In the presence of EDTA more sulphydryl groups react with HEDD, the rate of this reaction being dependent on the concentration of EDTA. Whereas 10 mM-EDTA rapidly makes practically all SH groups available to HEDD, in the presence of 1 mM-EDTA one hour of incubation is usually necessary for reaching the same endpoint. The presence of ATP

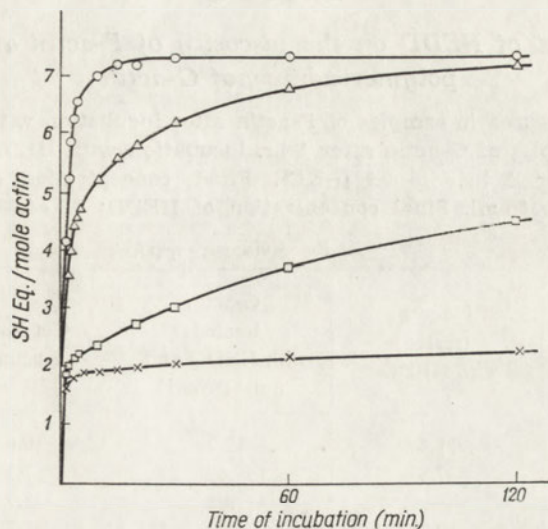


Fig. 1. The reaction of sulphydryl groups of G-actin with HEDD. Actin was incubated with HEDD at room temp.; at the time intervals indicated in the Figure the extinction values were read in the spectrophotometer. The reaction mixture contained G-actin from which free ATP was removed by treatment with Dowex 1 suspension (final concentration of actin 0.7 mg. per ml.), 0.3 mM-HEDD and 2.0 mM-tris-HCl, pH 7.0. If EDTA alone or with ATP was present these reagents were added to G-actin 5 min. before addition of HEDD. (X), Without EDTA; (Δ), 1 mM-EDTA present; (O), 10 mM-EDTA present; (□), 1 mM-EDTA and 1 mM-ATP present.

in equimolar concentration to EDTA leads only to a retardation of the process of rendering SH groups accessible to HEDD. After about 4 hr. of incubation of G-actin with HEDD in the presence of both 1 mM-EDTA and 1 mM-ATP the same endpoint (not shown in the Figure) has been reached as in the absence of ATP. In the presence of EDTA a mean value of about 7 SH Eq. reacting with HEDD per mole of actin was obtained.

The same number of SH equivalents has been found in heat denatured G-actin. In this case as well as in the presence of EDTA the obtained values varied in experiments from 6.7 to 7.5 SH Eq. per mole of actin.

After polymerization of G-actin to F-actin the number of sulphydryl groups directly reacting with HEDD and the rate of the reaction do not change at all; the same mean value 2.0 Eq. of SH groups per 60 000 g. of F-actin has been found as in the case of G-actin. On the other hand,

the addition of 1 mM-EDTA to F-actin does not increase the number of sulphhydryl groups available to HEDD.

The action of HEDD leads neither to the loss of polymerizability of G-actin nor to the depolymerization of F-actin (Table 1). These results

Table 1

The effect of HEDD on the viscosity of F-actin and on the polymerizability of G-actin

Viscosity was measured in samples of F-actin after incubation with HEDD for 1 hr. at 20° and in samples of G-actin after 1 hr. incubation with HEDD and subsequent polymerization for 2 hr. in 0.1 M-KCl. Final concentration of actin: 0.06 to 0.10 g/100 ml. Final concentration of HEDD: 0.3 - 0.35 mM.

Expt. no.	Reduced viscosity η_{sp}/c		
	F-actin treated with HEDD	G-actin treated with HEDD and polymerized	Control F-actin
1	11.4	12.7	10.6
2	7.8	—	7.3
3	—	9.1	8.7
4	10.0	—	11.7

show that those SH groups which react directly with HEDD are not involved in the polymerization of actin. As can be seen from Table 2 the reaction with HEDD does not also cause any release of bound ATP of G-actin. All these observations seem to suggest that HEDD reacts

Table 2

The effect of HEDD on the bound ATP of G-actin

G-actin, labelled with [¹⁴C]ATP was treated with Dowex 1 suspension (see Methods). The obtained actin solution (1.0 to 1.5 mg/ml.) was incubated with 0.3 mM-HEDD in 2 mM-tris, pH 7.0. At time intervals indicated in the Table the samples of the reaction mixture were treated with Dowex 1 suspension in 2 mM-tris - HCl, pH 8.0. After removal of resin radioactivity and protein concentration were determined.

Expt.	Incubation time (min)	Bound ATP (counts/min./mg. protein)	
		HEDD-treated actin	Control actin
I	3	1540	1480
	30	1330	1500
II	20	1358	1345
	40	1256	—
	60	1210	1210

Table 3

The effect of pretreatment of actin with NEM on the reaction of SH groups with HEDD

One part of G-actin solution was treated with 1 mM-NEM in 10 mM-tris - HCl, pH 7.0; the second one was used as a control. After 1 hr. both solutions were polymerized in 0.1 M -KCl for 2 hr. and then centrifuged in Spinco for 3 hr. at 90 000 *g* [21]. Pellets were homogenized in 0.2 mM-ATP in 2 mM-tris - HCl, pH 8.0, and subsequently dialysed for 40 hr. against the same solution. In the NEM-treated G-actin and in the control G-actin SH groups as well as polymerizability after the reaction with HEDD were measured.

Actin	Number of SH groups per 60 000 g. of actin		Reduced viscosity (η_{sp}/c) after polymerization			
	Directly reacting with HEDD		After denaturation by:		directly	1 hr. after treatment with HEDD
	in G-form	in F-form	EDTA	heat		
NEM-treated	0.14	0.23	4.95	4.90	6.3	7.2
Control	2.08	—	6.95	—	7.2	—

with the same sulphydryl groups which react with NEM. In order to prove this assumption the effect of HEDD was checked on actin previously treated with NEM. Table 3 shows that after treatment with NEM neither G-actin nor F-actin contain additional sulphydryl groups directly reacting with HEDD. Moreover, in the presence of EDTA, or after heat denaturation, in actin pretreated with NEM only about 5 SH Eq. per mole of actin could be found in comparison to about 7 found in the control. The subsequent addition of HEDD to NEM-treated actin does not also change its polymerizability. These results indicate that HEDD and NEM react with the same sulphydryl groups of actin molecule.

DISCUSSION

The effect of disulphides on sulphydryl groups of actin and on the properties of this protein have not been studied so far. Feuer *et al.* in an earlier paper [10] mentioned only the oxidative effect of cystine on G-actin and its inhibitory effect on the polymerization of this protein.

In the present paper HEDD was used for the study of reactivity of SH groups of actin towards disulphides. This reagent has several advantages. First, it is highly reactive because of the asymmetric structure and secondly, the product of its reaction with sulphydryl groups (dinitrothiophenol) is characterized by a peak at 408 m μ and by a high molar extinction coefficient.

The results presented in this paper show that HEDD reacts with two SH groups of native actin molecule. The rate of the reaction of HEDD with these SH groups is very high and is similar to that of the reaction with such low molecular weight SH-compounds as cysteine and glutathione [3, 4]. This fact supports the observation of other authors [14, 23, 24] that two SH groups of actin are easily accessible to sulphydryl reagents. No influence of ATP on the rate of the reaction of these SH groups with HEDD could be found in contrast to its protective effect against the reaction of other SH groups of actin molecule with mercurials [7, 24]. As mentioned above the results of the present experiments seem to support the view that NEM and HEDD react with the same SH groups of actin. The reaction of actin with either of these reagents leads neither to a loss of polymerizability nor to a release of bound nucleotide. Moreover, native actin in which two SH groups had been previously blocked by NEM, does not react anymore with HEDD. The role of these peculiar SH groups of actin remains unknown; they are, however, extremely chemically active as is shown by the reactions with NEM and HEDD, by an instantaneous reaction with mercurials [14, 23, 24] and by the availability to autooxidation. Somewhat surprisingly, it has been observed quite recently (W. Drabikowski, unpublished) that during the reaction of G-actin with HEDD some release of bound calcium takes place. This would indicate that in certain conditions the dissociation of bound calcium may occur without a release of bound ATP.

It is known that the action of EDTA leads to the release of bound calcium and bound ATP and to inactivation of G-actin [2, 11, 19, 26, 23]. As a result of this process all SH groups become available to NEM [23, 24] and, according to the present experiments, to HEDD. Maruyama & Martonosi [20] observed that ATP prevents the denaturing effect of EDTA. The present results show, however, that at least in the presence of HEDD free ATP only retards the effect of EDTA.

It is generally accepted that EDTA has no effect on F-actin [2, 11]. In their recent paper Kasai & Oosawa [13] have reported that the treatment of F-actin with EDTA increases the number of SH groups rapidly reacting with PCMB from 2 to 4 without causing depolymerization of F-actin. In the present work, however, the presence of EDTA did not lead to any increase of SH groups of F-actin reacting with HEDD.

Table 4 summarizes the data of different authors concerning the total number of SH equivalents in actin molecule. In the earlier papers a value of 4 to 5 titratable equivalents of sulphydryl groups per mole of actin was found [25, 15, 1, 2]. Since amino acid analysis of actin gave the value of about 6 to 7 half-cystine residues per mole [12, 15] it was even suggested that actin molecule contained one disulphide bridge [17]. The recent results of Katz & Mommaerts [14] and Tonomura & Yoshimura [24] as well as the results presented here show, however, that this is

not the case, since the number of chemically determined SH groups corresponds to that obtained on the basis of amino acid analysis. Table 4 shows that the mean values for the number of SH groups obtained recently [14, 24] amount to about 6.5 Eq. per mole of actin. In consequence the value of 6 was usually accepted, although Katz & Mommaerts [14] pointed out the possibility of existence of 7 SH Eq. in actin molecule.

Table 4

The total number of SH equivalents per 60 000 g. of actin

Method of determination	Number of SH groups	Author
Amino acid analysis	6.0	Ivanov I.I. <i>et al.</i> [12]
	6.8	Kominz D.R. <i>et al.</i> [15]
	6.7	Carsten H.E. [6]
Titration with: NEM	4.2	Tsao T.C. <i>et al.</i> [25]
CH ₃ HgNO ₃	4.4	Kominz D.R. <i>et al.</i> [15]
CH ₃ HgNO ₃	4.0	Bárány M. [1]
CH ₃ HgNO ₃	4.7 - 5.0	Bárány M. <i>et al.</i> [2]
Amperometric titration with: Ag ⁺	6.0 - 7.0	Katz A.M. <i>et al.</i> [14]
Hg ²⁺ or Ag ⁺	5.0 - 6.0	Poglavov B.F. <i>et al.</i> [22]
Spectrophotometrically with: NEM	8.3	Strohm R.C. <i>et al.</i> [23]
PCMB	6.1 - 6.8	Tonomura Y. <i>et al.</i> [24]
PCMB and NEM	6.0 - 7.0	Katz A.M. <i>et al.</i> [14]
HEDD	6.7 - 7.5	This paper

The results presented in this paper also indicate that G-actin contains 7 SH Eq. per mole. The values lower than 7 for the total number of SH Eq. and, correspondingly, the values lower than 2 for directly reacting SH groups obtained in this paper in some cases, especially in older actin preparations, were most probably due to a slight autooxidation of SH groups [14]. The differences, however, between the total number of SH groups and the number of directly reacting SH groups amounted in each experiment to about 5 and were not lower than 4.6, indicating that rather only the directly reacting SH groups were easily autooxidizable.

The authors wish to thank Prof. W. Niemierko for his interest and criticism in the course of this work.

SUMMARY

The effect of β -hydroxyethyl-2,4-dinitrophenyl disulphide (HEDD) on sulphydryl groups of actin has been investigated. This compound reacts with two sulphydryl groups of native actin molecule which are

involved neither in polymerization nor in nucleotide binding. These groups correspond to those reacting with *N*-ethylmaleimide.

The total number of SH groups in actin determined using HEDD after heat denaturation or in the presence of EDTA amounts to 7.

REFERENCES

- [1] Bárány M., in *Symposium on Sulfur in Proteins* (Benesch R., Benesch R. E., Boyer P. D., Klotz J. M., Middlebrook W. R., Szent-Györgyi A. G. & Schwarz D. R., eds.) p. 317. Academic Press, New York 1959.
- [2] Bárány M., Nagy B., Finkelman F. & Chrambach A. - *J. Biol. Chem.* **236**, 2917, 1961.
- [3] Bitny-Szlachto S. - *Acta Polon. Pharm.* **17**, 373, 1960.
- [4] Bitny-Szlachto S., Kosiński J. & Niedzielska A. - *Acta Polon. Pharm.* **20**, 365, 1963.
- [5] Böhme H. & Stachel M. D. - *Z. Anal. Chem.* **154**, 27, 1957.
- [6] Carsten M. E. - *Biochemistry* **2**, 32, 1963.
- [7] Drabikowski W. & Gergely J. - *J. Biol. Chem.* **237**, 3412, 1962.
- [8] Drabikowski W. & Gergely J. - *J. Biol. Chem.* **238**, 640, 1963.
- [9] Drabikowski W. & Bitny-Szlachto S. - *Bull. Acad. Polon. Sci., Ser. Biol.* **11**, 165, 1963.
- [10] Feuer G., Molnar F., Pettko E. & Straub F. B. - *Hung. Acta Physiol.* **1**, 150, 1948.
- [10a] Gornall A. G., Bardawill C. I. & David H. M. - *J. Biol. Chem.* **177**, 751, 1949.
- [11] Grubhofer N. & Weber H. H. - *Z. Naturforsch.* **16b**, 435, 1961.
- [12] Ivanov I. I. & Asmolowa E. N. - *Biokhimiya* **15**, 201, 1950.
- [13] Kasai M. & Oosawa F. - *Biochim. Biophys. Acta* **75**, 223, 1963.
- [14] Katz A. M. & Mommaerts W. F. H. M. - *Biochim. Biophys. Acta* **65**, 82, 1962.
- [15] Kominz D. R., Hough A., Symonds P. & Laki K. - *Arch. Biochem. Biophys.* **50**, 148, 1954.
- [16] Kuschinsky G. & Turba F. - *Biochim. Biophys. Acta* **6**, 426, 1951.
- [17] Laki K. & Standaert J. - *Arch. Biochem. Biophys.* **86**, 16, 1960.
- [18] Martonosi A., Gouvea M. A. & Gergely J. - *J. Biol. Chem.* **235**, 1700, 1960.
- [19] Martonosi A. & Gouvea M. A. - *J. Biol. Chem.* **236**, 1345, 1961.
- [20] Maruyama K. & Martonosi A. - *Biochem. Biophys. Res. Comm.* **5**, 85, 1961.
- [21] Mommaerts W. F. H. M. - *J. Biol. Chem.* **198**, 459, 1952.
- [22] Poglazov B. F. & Baev A. A. - *Biokhimiya* **26**, 535, 1961.
- [23] Strohmman R. C. & Samorodin A. J. - *J. Biol. Chem.* **237**, 363, 1962.
- [24] Tonomura Y. & Yoshimura J. - *J. Biochem. (Tokyo)* **51**, 259, 1962.
- [25] Tsao T. C. & Bailey K. - *Biochim. Biophys. Acta* **11**, 102, 1953.
- [26] Yoshimura J. & Tonomura Y. - *J. Biochem. (Tokyo)* **50**, 79, 1961.

BADANIA NAD GRUPAMI SULFHYDRYLOWYMI AKTYNY

Streszczenie

Badano wpływ dwusiarczku β -hydroksyetylo-2,4-dwunitrofenylowego (HEDD) na grupy sulfhydrylowe aktyny. Związek ten reaguje z dwoma grupami SH cząsteczki rodzimej aktyny, nie biorącymi udziału w procesie polimeryzacji, ani w wiązaniu nukleotydów przez aktynę. Wyniki wskazują na to, że są to te same grupy SH, które reagują z *N*-etylomaleimidem.

Received 8 February 1964.

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**BEHAVIOUR OF SOME PHOSPHORUS COMPOUNDS
AND CARBOHYDRATES IN THE WAX MOTH DURING ANOXIA
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It is well known that general features of insect metabolism resemble those of the higher animals. However, some of the metabolic pathways seem to occur only in insects and some of the chemical constituents of the body are present only in certain groups of insects and, sometimes, even in single species. As a peculiar example trehalose can be given which highly accumulates in the haemolymph of many, perhaps most, insects as the chief carbohydrate [37] and is only exceptionally found in other animals. The formation of large amounts of pyrophosphates was described in *Celerio euphorbiae* (Heller et al. [12]) and intense formation of pyro- and polyphosphates was found in *Galleria mellonella* and *Achroea grisella* (Niemierko & Niemierko [21, 23]).

The processes of anaerobic glycolysis which are universally found in the animal kingdom appear to be somewhat peculiar in insects. In experiments performed on isolated thoracic muscle of *Periplaneta americana* and *Locusta migratoria* Kubišta [15, 16] and Bücher & Klingenberg [3] found that during anoxia α -glycerophosphate is formed as the chief endproduct of glycolysis. In jumping muscles of insects the metabolism is, however, similar to that of mammalian muscles and mainly lactic acid is produced. The formation of glycerophosphate, although in smaller amounts and only in the first period of anoxia, was recently described [14] in housefly. Price [27] suggested that α -alanine accumulates in the housefly in an atmosphere of nitrogen. In pupae of *Cecropia* [32] during anoxia glycerol is formed, while in the pupae of *Cynthia samia* lactic acid was the endproduct of glycolysis and its amount was proportional to the duration of anoxia. In the last mentioned insect glycerol increased only slightly but at a reduced temperature its amount during anoxia was considerably higher. Chino [5] found in diapauzing eggs of the silkworm transformation of glycogen into sorbitol and glycerol.

In some insects other endproducts of glycolysis, besides those

mentioned above, are present: succinic, acetic, malic acids. But even together with lactic and pyruvic acids they often are not sufficient to balance the carbohydrates disappearing.

The relation between the amount of different endproducts of glycolysis evidently depends on the activity of particular enzymes engaged in metabolic processes and hence striking discrepancies can be found in various groups of insects (c.f. Gilmour [9]). According to Zebe & McShan [39] and Chefurka [4] glycerophosphate dehydrogenase is in muscles of insects much more active than lactic acid dehydrogenase. As a result, glycerophosphate and not lactic acid is formed in the muscles of most of the insects as the chief product of anaerobic glycolysis with utilization of phosphates split from ATP and phosphagen.

On the other hand, our earlier investigations have shown that in larvae of *Galleria mellonella* when they are put in anaerobic conditions very considerable amounts of orthophosphate accumulate in the body and disappear during postanoxic recovery in air. A similar phenomenon was described by Winteringham [33] in *Musca domestica* and confirmed thereafter in the same laboratory [14, 28].

The aim of the present investigation was (1) to study more closely the phosphorus metabolism during anaerobiosis of larvae, pupae and adults of *Galleria mellonella*; (2) to investigate the carbohydrate metabolism during anoxia; (3) to examine some of the biochemical processes which take place after anaerobiosis during recovery in air.

A preliminary account of some of the results has appeared [20, 22].

MATERIAL AND METHODS

The experiments were performed on *Galleria mellonella* L. bread at 30° as described earlier [24]. In most of the experiments fully grown larvae, weighing usually from 140 to 200 mg. each, were used; for some experiments pupae (males) of a definite age were taken, and in a few cases freshly hatched adults (males) were investigated.

The insects were taken in groups of individuals and put in 50 ml. tubes (with two stopcocks) through which a strong stream of nitrogen from a gas cylinder was blown during a few minutes to remove all traces of the air. The vessels were then immediately tightly closed and kept at 30° for various periods of time from 10 min. to 13 hr. Afterwards, the insects from some of the vessels were analysed directly whereas those from other vessels were kept in air at 30° for various periods of time in order to study the processes which occur during recovery. The insects which served as control were kept in air also at 30° usually for the same periods of time as the experimental animals.

In experiments which were performed on larvae they were previously

starved during one to two hours because it appeared that such animals better sustain the lack of oxygen.

In some experiments the larvae were ligated just behind the head and were in this way immobilized [25]. This treatment seemed to be important as the movements in the control larvae, and in those which recovered in air after anaerobiosis, could have an influence on the metabolism.

The acid soluble phosphorus compounds were analysed after extraction with TCA or HClO_4 , as described earlier [19]. For a rough determination of labile and of nonhydrolysable phosphates a fractionation with N-HCl at 100° according to the method described in an earlier paper [19] was performed. Phosphoarginine (PA^1) was determined according to Rosenberg *et al.* [29] and the sum of ATP and ADP according to Slater [30]. Beside this ATP, ADP and AMP were separated by column chromatography in a cold room and assayed according to Cohn & Carter [6]; the separation was checked by paper chromatography. Phosphorylcholine, phosphorylethanolamine and glycerophosphate were determined as described earlier [17]. Glycerol was estimated after paper chromatographic separation with a microiodometric method of W. Niemierko (unpublished). Lactic acid was determined according to Barker & Summerson [1] and pyruvic acid according to Friedman [8]. The carbohydrates were determined by the anthrone method; glycogen was precipitated by alcohol from the cold as well as from the hot TCA extract; trehalose was determined after elimination of glycogen and after destruction of monosaccharides with NaOH [37]; the oxygen consumption of the larvae before the experiments and during the recovery period was determined in a conventional Warburg apparatus.

RESULTS

Biological observations

Preliminary experiments indicated that about 25% of the larvae survived after 36 hr. anaerobiosis, about 50% survived when deprived of oxygen for 12 hr., and practically all larvae remained alive when anaerobiosis did not last longer than 6 hr. Put into the normal conditions at 30° the larvae gradually began to feed and underwent metamorphosis. Compared with the control animals the metamorphosis started with a certain delay, although it did not last longer. The pupae appeared to be more resistant towards anaerobiosis than the larvae, whereas the

¹ Abbreviations used: PA, phosphoarginine; P_{10} , phosphate split after 10 min. hydrolysis in 1 N-acid at 100° , minus phosphoarginine; P_{180} , phosphate split after 180 min. hydrolysis, minus labile phosphates; P_{nh} , nonhydrolysable phosphates; $\text{P}_{\text{t.ac.sol.}}$, total acid soluble phosphates; TCA, trichloroacetic acid.

adults never survived if they were deprived of oxygen for longer than 8 to 10 hr. The differences in susceptibility to lack of oxygen of pupae of different age were also described by Parok & Buck [26].

In a few seconds after blowing the stream of nitrogen through the vessels spontaneous movements of the larvae, even of those previously immobilized by ligaturing, and of the pupae became visible. These movements usually lasted for 10 to 15 sec. most probably until the last traces of oxygen were eliminated.

After anaerobiosis the insects were quite motionless for some time; they seemed to be inexcitable, as they did not react when touched with a needle. The longer was the period of anaerobiosis the longer lasted the inexcitability, sometimes up to 12-24 hr. after 15 hr. of lack of oxygen.

Phosphorus compounds

In the larvae of *Galleria mellonella* which are kept in nitrogen at 30° the amount of P_i in the body increases continuously. Data in Fig. 1 show that after about 12 hr. of anaerobiosis the larvae contain 3 times more P_i

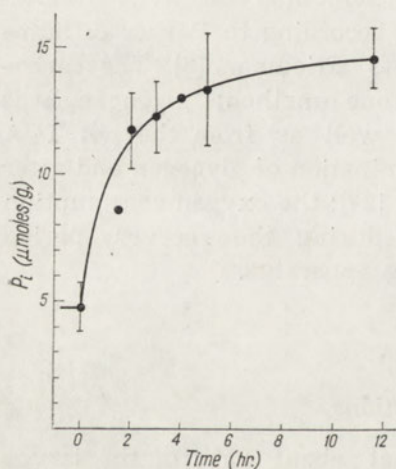


Fig. 1. The increase of orthophosphate in larvae of wax moth during anoxia. Each point represents mean value, \pm S.D., from 3 to 16 experiments.

than the control ones. The increase of P_i was recognizable already during the first few minutes. So for instance in some of experiments the lack of oxygen which lasted only 10 min. produced an increase of P_i by about 30% over the mean normal value.

These results explain our earlier observations [19] in which vacuum drying of wax moth was found to cause a considerable increase of orthophosphate. This increase of P_i occurred at the expense of a partial breakdown of some acid soluble phosphorus compounds. Hence it may be understood that the efficient cause of observed accumulation of orthophosphate was not the drying but the lack of oxygen. In a few of

experiments we were now able to show that splitting of some phosphates with the liberation of P_i occurs both in vacuum and in an atmosphere of nitrogen.

During recovery in air the amount of orthophosphate in the larvae gradually diminishes. The rate of this process as illustrated in Table 1 depends upon the duration of anaerobiosis. Experiments performed to elucidate which constituents of the larval body undergo dephosphorylation during anaerobiosis showed (Table 2) that P_i which was formed

Table 1

Orthophosphate in larvae of wax moth during anoxia and recovery in air

Mean values, \pm S.D., are given; in parentheses the number of experiments.

Experimental conditions		P_i	
Duration of anoxia (hr.)	Recovery in air (hr.)	$\mu\text{moles/g.}$	% of initial value
0 (Control)		5.34 ± 0.26 (10)	100 ± 4.8
3	0	12.0 ± 1.36 (5)	225 ± 11.2
3	2	6.2 ± 0.71 (5)	117 ± 11.3
5	0	14.5 ± 2.39 (8)	272 ± 16.5
5	1	10.6 (2)	198
		10.4; 10.8	
5	2	9.0 ± 1.19 (3)	169 ± 13.2
5	3	8.5 ± 0.88 (3)	159 ± 10.3
12	0	15.4 (1)	332
12	3	13.2 (1)	247

Table 2

Orthophosphate, phosphoarginine and P_{10} in larvae of wax moth during anoxia

Each determination in duplicate. The results are expressed as $\mu\text{moles/g.}$

Duration of anoxia (hr.)	P_i		PA		P_{10}		$P / + 1$	
	Contents	Difference after anoxia	Contents	Difference after anoxia	Contents	Difference after anoxia	Contents	Difference after anoxia
0	4.27	—	1.99	—	5.69	—	7.68	—
1.5	8.60	+4.33	1.03	-0.96	2.45	-3.24	3.48	-4.20
3	13.60	+9.33	0.37	-1.62	2.05	-3.64	2.42	-5.26

during 1.5 hr. corresponded to the amount of labile phosphates disappearing from the body (P_A and P_{10}). The degree of accumulation of P_i during a more prolonged anaerobiosis indicated, however, that in this case also some other phosphates were split.

Table 3 summarizes the results of experiments in which anoxia lasted up to 13 hr. During the first 3 hr. the amount of labile phosphates diminished to a very low level but further decrease was no longer considerable.

Table 3

Acid soluble phosphorus compounds in larvae of wax moth during anoxia

Mean values \pm S.D., are given. The results are expressed as μ moles/g.

Duration of anoxia (hr.)	No. of expts.	P_i	P_A	P_{10}	P_{180}	P_{nh}^*	$P_{t.ac.sol.}^{**}$
0	8	5.33 ± 0.49	2.03 ± 0.70	5.92 ± 1.07	4.61 ± 0.27	16.3 ± 1.99	33.8 ± 1.22
3	4	12.3 ± 1.83	0.84 ± 0.21	1.63 ± 1.09	2.84 ± 0.73	12.0 ± 5.60	33.7 ± 3.59
5	4	13.0 ± 1.07	0.45 ± 0.24	1.52 ± 0.14	2.41 ± 0.95	19.2 ± 1.28	36.8 ± 0.01
13	3	18.4 ± 2.19	0.76 ± 0.26	0.96 ± 0.23	2.86 ± 0.83	14.9 ± 1.77	39.2 ± 1.13

* The differences between the normal value and those after anoxia are not significant.

** The difference between the normal value and that after 5 hr. of anoxia is significant at $P < 0.05$, and after 13 hr. of anoxia at $P < 0.01$.

The quantity of nonhydrolysable phosphates is highly variable. According to Lenartowicz *et al.* [17] in larvae of *Galleria mellonella* this fraction chiefly consists of phosphorylethanolamine, phosphorylcholine and glycerophosphate. The behaviour of these compounds in anoxia deserves a more detailed examination. Some of the preliminary results are discussed below.

The total amount of acid soluble phosphates did not change during the first hours of anaerobiosis. In experiments, however, in which the larvae had been deprived of oxygen for 13 hr. the amount of these compounds distinctly increased. At the same time a certain amount of phospholipids (10 to 20%) was split. This point deserves, however, further investigation, as well as the question whether other acid insoluble phosphorus com-

pounds also are engaged in the processes which take place in the larvae during anaerobiosis.

As has been known from our earlier papers in larvae of *Galleria mellonella* large amounts of inorganic pyro- and tripolyphosphates are formed and eliminated with the excreta [23]. These substances belonging to labile phosphates were in the experiments presented in Table 3 determined as P_{10} together with the nucleoside polyphosphates (ATP, ADP). It was therefore necessary to differentiate the behaviour during anoxia of these two kinds of compounds. A tentative calculation of the quantity of pyro- and tripolyphosphates was made from the difference between the total P_{10} and the sum ATP + ADP estimated according to Slater [30]. The figures obtained were highly variable. It must be stressed, however, that the greater part of inorganic polyphosphates is present in the wax moth in the Malpighian tubes and in the hindgut in the form ready to be excreted. It seems therefore unlikely that this large part of the polyphosphates could be involved in the metabolism at all. On the other hand, it deserves a special investigation using specific methods of determination to elucidate whether the very small part of these compounds present in the tissues (cf. [23]) may be hydrolysed during anoxia.

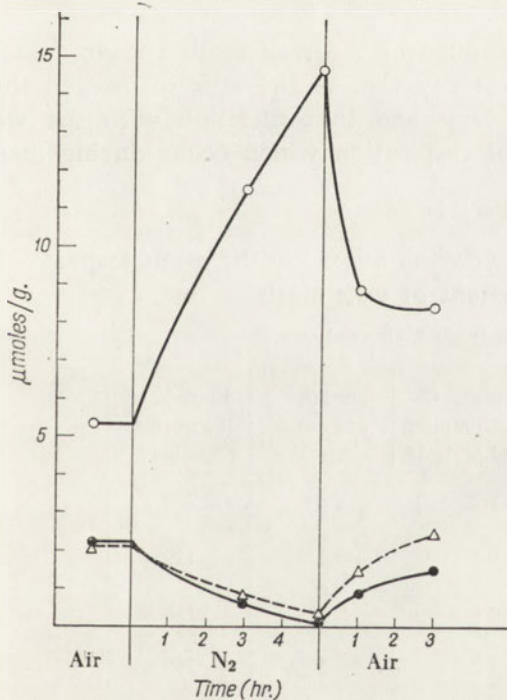


Fig. 2

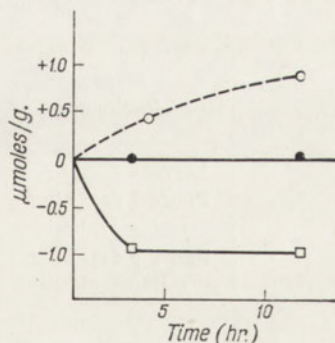


Fig. 3

Fig. 2. The change in the amounts of P_i , PA and (ATP + ADP) in larvae of wax moth during anoxia and recovery in air. (○), P_i ; (●), PA; (Δ), ATP + ADP.

Fig. 3. The change in the amounts of ATP and AMP in larvae of wax moth during anoxia. (○), AMP; (●), ADP; (□), ATP.

Fig. 2 illustrates the changes in the amount of P_i , PA and (ATP + ADP) during anaerobiosis as well as during postanoxic recovery in air. It can be seen that in nitrogen about 80% of nucleoside polyphosphates and even a somewhat larger quantity of PA were split during 5 hr. Three hours of postanoxic recovery were, however, sufficient for a complete resynthesis of the former and a resynthesis of about 60% of the latter. Data in Fig. 2 show at the same time that the large increase of P_i in anoxia must be connected with a hydrolysis also of some other phosphates and, correspondingly, the high diminution of P_i during recovery points to a partial resynthesis of these compounds.

In order to characterize the nucleotides more precisely, in some cases they were estimated according to Cohn & Carter [6]. The data in Fig. 3 which present mean values of analyses demonstrate that during anoxia ATP greatly diminishes and a certain amount of AMP is formed, whereas ADP remains practically unchanged. These results make highly probable that in the metabolism of ATP during anoxia ATPase and adenylate kinase participate. The presence of the last mentioned enzyme in insects was found by Gilmour & Calaby [10], and an increase of AMP during anoxia of the housefly was quite recently observed by Heslop *et al.* [14].

As was mentioned above, the pupa of the wax moth appeared to be more resistant and the imago less resistant to the lack of oxygen than the larva. In relation to these facts and in connection with the well known differences in the rate of respiration which occur during insect

Table 4

Increase of orthophosphate during anoxia at different stages of development of wax moth

Mean value from 2 - 3 analyses.

Stage of development	Normal O ₂ consumption (ml./g./hr.)*	Duration of anoxia (hr.)	P _i in % of the normal value
Larvae	1.2	3	230
Pupae 2 days after metamorphosis	0.5	3	129
Pupae 5 days after metamorphosis	0.8	3	150
Larvae	1.2	5	260
Pupae 5 - 6 days after metamorphosis	0.8 - 0.9	5	170
Larvae	1.2	2	180
Imago	3 - 8	2	268

* Data of Wojtozak [34].

development the experiments have been extended to pupae and to adults. The data obtained are summarized in Table 4; they show that the influence of anaerobiosis, as measured by the increase of P_i , is more evident in those stages of development which are characterized by a higher rate of metabolism.

Some preliminary experiments were performed to elucidate the influence of anoxia on the P_i increase in different tissues of *Galleria mellonella*. After 4 hr. of anaerobiosis the mean level of P_i in the whole larvae was more than twice higher (230%) than in control animals. At the same time the increase of P_i in the haemolymph appeared to be higher than in other tissues: it increased from 4 to 17 μ moles/g. (420%); simultaneously phosphorylethanolamine, phosphorylcholine and glycerophosphate, which are the main organic phosphates in the haemolymph, decreased.

Oxygen uptake during recovery in air

It was mentioned above that the processes of dephosphorylation which occurred under influence of lack of oxygen in the wax moth were to a great extent reversible and that during postanoxic recovery in air rephosphorylation prevailed. Thus, it can be calculated from the results presented in Table 3 and Fig. 2 that during 1 hr. of recovery in air a net synthesis of about 3 μ moles per 1 g. body weight of energy rich compounds (ATP and PA) took place and, besides this, a certain amount of some other P compounds was newly formed. It seemed therefore of interest to examine the utilization of oxygen by the larva of *Galleria mellonella* during the postanoxic recovery period.

Numerous data concerned with this question exist in the literature but they are not uniform. In some insects (*Tenebrio molitor*, *Euchytraideae*, *Periplaneta americana*) an oxygen debt always occurs after anoxia, while in some other insects (*Melolontha*, *Elateridae*) such phenomenon is not observable (cf. Gilmour [9]). It may be added that according to Rotta & Stannard (cf. [9]) oxygen debt is a feature peculiar to muscles, while in a number of other tissues it is not observed during postanoxic recovery.

In the present investigation the consumption of oxygen was measured in larvae which had been previously kept in nitrogen for 3 hr. The data in Fig. 4 show that during the first 60 to 90 min. of recovery in air the respiration of the larva was considerably lower than it was before anaerobiosis; it returned to the normal level not earlier than in about 2 hr.

Utilization of carbohydrates

It is a known fact that anaerobiosis is nearly universally connected with glycolysis, although the products of glycolysis may vary greatly. This is especially the case in insects [9].

Figure 5 illustrates the data found in the present investigation for the behaviour of carbohydrates. As can be seen, a sharp diminution of glycogen was visible after 3 hr. stay of the larvae in nitrogen; afterwards during the next 2 hr. of anoxia its amount diminished not very considerably. The behaviour of trehalose, the chief carbohydrate in the wax moth [38], was somewhat different: a distinct decrease of the amount of trehalose was observable only during a more prolonged anoxia. In the

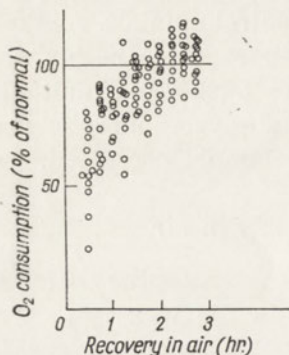


Fig. 4

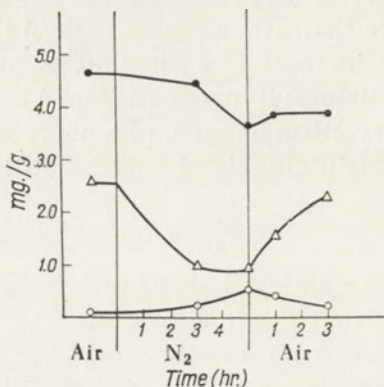


Fig. 5

Fig. 4. Oxygen consumption of larvae of wax moth during postanoxic recovery in air after 3 hr. of anaerobiosis. Each point represents a measurement on a single larva expressed in % of the value found in this animal before anaerobiosis.

Fig. 5. Concentration of glycogen, trehalose and lactic acid in larvae of wax moth during anoxia and recovery in air. (●), Trehalose; (Δ), glycogen; (○), lactic acid.

course of a very long period of anaerobiosis, up to 12 hr., a diminution of trehalose was still observable, but glycogen remained at a low level without any further change.

During 3 hr. of postanoxic recovery in air the amount of glycogen almost returned to the initial quantity (Fig. 5) while the quantity of trehalose (although it was partly resynthesized) was still lower than the normal value. It may be supposed that the difference in the behaviour of the two carbohydrates is connected with the distribution of these substances in the tissues. Glycogen is accumulated chiefly in the fat body [38], physiologically very active tissue, whereas by far the greater part of trehalose is found in the haemolymph. It would be therefore expected that glycogen is more easily accessible to the action of enzyme systems than trehalose chiefly present in the haemolymph, by far more inert tissue. The changes found in the haemolymph can be ascribed to processes which occur mainly, or even only then when haemolymph gradually comes into contact with particular tissues and their enzymes. The low level of glycogen which is found in the larva after a few hours

of anaerobiosis and which does not change further during a more prolonged anoxia may represent that part of this carbohydrate which is in a close connection with the structural elements of the cell. Such an opinion is in agreement with preliminary experiments from this laboratory (Lenartowicz, unpublished) in which it has been shown that under influence of low temperature glycogen falls to nearly the same low value (about 90 mg.%) as found in the present investigation and does not change further.

Figure 5 and Table 5 show the quantity of lactic and pyruvic acids, and of glycerol which were present in the larvae during anoxia. Although the relative amount of these substances was found to be highly increased, nevertheless, as their initial content in the body was extremely low, the total quantity accumulated in the larva could be responsible only for a not very considerable part of glycogen and trehalose which disappeared during anaerobiosis. It must be added that according to our preliminary analyses the content of monosaccharides, which is very low in the wax moth, practically does not change during anoxia.

Table 5

Carbohydrates and products of glycolysis in larvae of wax moth during anoxia

Mean values, \pm S.D., are given; in parentheses the number of experiments.

Duration of anoxia (hr.)	Glycogen* (mg. %)	Trehalose** (mg. %)	Lactic acid (mg. %)	Pyruvic acid (mg. %)	Glycerol (mg. %)
0	198 \pm 50 (6)	500 \pm 100 (6)	4.9 \pm 1.24 (7)	10.5 (2)	11.8 \pm 3.16 (7)
3	115 \pm 25 (6)	440 \pm 50 (6)	20.8 \pm 4.03 (4)	23.1 (2)	—
5	73 \pm 16 (9)	362 \pm 75 (9)	43.5 \pm 7.81 (4)	—	—
8	82 \pm 28 (4)	350 \pm 40 (4)	44.3 (2)	—	—
12	97 (2)	155 (2)	—	—	82.7 \pm 14.6 (3)

* The difference between the normal value and that after 3 hr. of anoxia is significant at $P < 0.05$, and the differences between normal and later data are significant at $P < 0.01$.

** The difference between the normal value and that after 3 hr. of anoxia is not significant; it is significant between the normal and later data: at $P < 0.05$ after 5 hr. of anoxia and at $P < 0.01$ later on.

In the present investigation likewise no increase of glycerophosphate was found in the larva. In some preliminary experiments, after several hours of anaerobiosis, analysis of particular larval tissues for phosphorus compounds was performed, but also in this case no increase of glycerophosphate could be found.

In this way the question of the products of glycolysis which are formed when the wax moth is put into anaerobic conditions still remains to be elucidated.

DISCUSSION

As has been shown in the present study the influence of experimental anoxia on the wax moth is connected with hydrolysis of different organic phosphates, in the first place of energy rich compounds, and in accumulation of large amounts of inorganic phosphate.

During postanoxic recovery in air a reversal of these processes takes place; P_i diminishes and the organic phosphates become reconstituted. It seems to be remarkable that oxygen consumption is at the beginning of recovery period highly reduced and rises gradually to the normal value.

The interesting observation that during the pupal period of *Celerio euphorbiae* the rate of respiration was inversely proportional to the amount of P_i in the body was made by Heller & Mokłowska already in 1930 [13]. Later on similar results for P_i and a reverse relationship for easily hydrolysable P were found by Levenbook in the blowfly [18]. These facts according to Wyatt [35] indicate that during diapause the metabolism is limited by some factors, and inorganic phosphate accumulates as a result.

It is plausible to consider experimental anaerobiosis as an extreme case of insufficient oxygen supply. Some similarities may exist between different metabolic processes such as occur during hypoxia, anoxia, inadequate oxygen supply, and depression of respiration as in pupal diapause. Nevertheless, one must realize that many important features of metabolism can be entirely different in particular cases. Three such cases will be discussed as examples.

(1), Intense muscular activity in vertebrates during which the normal oxygen supply becomes insufficient and which is accompanied by typical anaerobic glycolysis. As a rule, except in the most severe circumstances, orthophosphate is not accumulated; but during recovery an oxygen debt is normally observable. However, in some insects which utilize fats as fuel during flight, e.g. the migratory locust, no oxygen debt is found.

(2), Low metabolism and reduced respiration in the middle of insect metamorphosis, esp. in the pupal diapause. The extremely complicated control mechanisms involved in these processes are undoubtedly connected with the activity of insect hormones which have been intensively investigated during the last several years [cf. 11, 36] and perhaps also with activity of a hormone found quite recently in the hindgut of larvae of a corn borer, *Ostinea nubilialis* and in larva of *Galleria mellonella* [2]. The coincidence between the low oxygen consumption, decrease of labile phosphates and an increase of orthophosphate, which is found in the middle of metamorphosis [36], may reflect only a small part of events precisely regulated and still not very well known so far.

(3), The experimental anoxia and the postanoxic recovery of the wax moth. This last example seems to be somewhat less complicated than the two previous ones and it is perhaps possible to give at least a tentative explanation of some of the results obtained.

It seems likely that the hydrolysis of energy rich phosphates and other phosphorus compounds followed by accumulation of P_i , as found in the present investigation during anaerobiosis, is the result of injury of some cell constituents. de Duve in his lysosome concept [7] mentions that under influence of anoxia intracellular rupture of lysosomes and intracellular release of enzymes may occur causing generalized autolysis. Various hydrolytic and glycolytic processes are initiated in this way or enhanced and, as one can suppose, some of them may proceed not only in the injured cells but also outside the cells. It is possible that the released enzymes can get into the haemolymph and under their influence phosphorylethanolamine and phosphorylcholine and other nonhydrolysable phosphates present in very considerable amounts [17] are split and, as has been shown, larger amounts of inorganic phosphate than in any other tissue accumulate. It seems to be important to mention that our preliminary results indicate that the amounts of P_i and other phosphates do not change in the haemolymph taken from normal respiring larvae and kept *in vitro* under nitrogen during several hours.

At the beginning of postanoxic recovery in air the larval tissues contain an increased amount of orthophosphate and AMP. At the same time the quantity of some organic phosphates, especially ATP and PA , is greatly diminished. It is difficult so far to understand what is the mechanism which is responsible for the reduced respiration rate which is observable during the first one or two hours of the recovery period. In any case the gradual increase of respiration seems to go parallel with the diminution of P_i and the resynthesis of energy rich phosphates.

From experiments performed on isolated mitochondria it is well known that oxidative phosphorylation during which various substrates are oxidized depends on the presence of P_i and ADP. There is no deficiency of these substances after anaerobiosis, the amount of P_i is even highly increased. However, according to Slater & Holton [31] AMP is phosphorylated only slowly and this process probably requires first formation of ATP from ADP and secondly sufficient adenylate kinase to promote phosphorylation of AMP. It seems to be possible therefore that accumulation of AMP during anoxia, found in the present investigation, may be partly responsible for the low initial oxygen uptake by the larvae during recovery in air.

Some data in the literature point to a harmful effect of extra quantities of orthophosphate in the body. Our unpublished results indicate that when larvae of *Galleria mellonella* are fed with highly increased amounts

of P_i a retention of P_i in the body takes place and, at the same time, the processes of growth appear to be delayed.

There exist many observations which show that the body activity and especially the muscle activity are related to the amount of ATP and phosphagen. The results of the present investigation are in good agreement with these observations. As has been shown, the larvae after anoxia appear to be completely motionless. They begin to move only after a certain period of recovery. This corresponds approximately to the time in which the energy rich phosphates are gradually resynthesized.

In this way it seems possible that in the course of the processes of postanoxic recovery the high concentrations of P_i , increase of AMP and the diminished amount of energy rich compounds may serve as the limiting factors for respiration together with the participation of many more other substances whose role remains to be elucidated.

We thank Mrs. W. Chmurzyńska for determinations of oxygen consumption, Mrs. B. Ginter for column chromatography of nucleotides, and Mrs. M. Krzyżanowska and Mrs. B. Rudzisz for skilful technical assistance.

SUMMARY

1. The influence of anoxia was studied on larva of the wax moth. When the animals were kept in nitrogen at 30° for up to 13 hr. the inorganic phosphate gradually accumulated from about 4 to about 15 μ moles per 1 g. body weight. At the same time the amount of some organic phosphates, in the first place ATP and phosphoarginine, diminished while AMP increased and ADP did not change. The quantity of glycogen was highly reduced already during the first few hours of anoxia from about 200 to 80 mg.%; later on this small quantity remained without any further change. Trehalose started to decrease somewhat later than glycogen; during a prolonged anoxia it diminished from 500 to about 200 mg.%. No increase of glycerophosphate and monosaccharides was observed. Lactic and pyruvic acids and glycerol increased but their total quantity was too small to account for the disappearance of glycogen and trehalose.

2. During the postanoxic recovery in air the accumulated products diminished and the compounds which had been split became reconstituted. At the beginning of recovery the oxygen uptake was considerably reduced; it increased to the normal value only in about 2 hr.

3. Some of the processes which take place in the wax moth during anoxia are discussed in relation to the lysosome concept of de Duve. Besides, it is suggested that the accumulation of orthophosphate and AMP and the decrease of ATP may be partly responsible for the reduced respiration at the beginning of the recovery period.

REFERENCES

- [1] Barker S. B. & Summerson W. H. - *J. Biol. Chem.* **138**, 535, 1941.
- [2] Beck S. D. & Alexander N. - *Science* **143**, 478, 1964.
- [3] Bücher T. & Klingenberg M. - *Angew. Chemie* **70**, 552, 1958.
- [4] Chefurka W. - *Biochem. J.* **28**, 660, 1958.
- [5] Chino H. - *J. Insect. Physiol.* **2**, 1, 1958.
- [6] Cohn W. E. & Carter C. E. - *J. Am. Chem. Soc.* **72**, 4273, 1950.
- [7] de Duve C., *The lysosome concept*. In *Lysosomes* (A. V. C. de Reuck & M. P. Cameron, eds.) J. A. Churchill, Ltd, London, 1963.
- [8] Friedman T. E., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) Vol. 3, p. 414. Acad. Press. N.Y., 1957.
- [9] Gilmour D., *Biochemistry of Insects*. Acad. Press. N.Y., 1961.
- [10] Gilmour D. & Calaby J. H. - *Enzymologia* **16**, 34, 1953.
- [11] Harvey W. R. - *Ann. Rev. Entomol.* **7**, 57, 1962.
- [12] Heller J., Karpiak S. & Zubikowa J. - *Nature* **166**, 187, 1950.
- [13] Heller J. & Mokłowska A. - *Biochem. Z.* **219**, 473, 1930.
- [14] Heslop J. P., Price G. & Ray J. W. - *Biochem. J.* **87**, 35, 1963.
- [15] Kubišta V. - *Nature* **180**, 549, 1957.
- [16] Kubišta V. - *Marburger Sitzungsber.* **81**, 17, 1961.
- [17] Lenartowicz E., Rudzisz B. & Niemierko S. - *J. Insect. Physiol.* **10**, 89, 1964.
- [18] Levenbook L. - *J. Cell. Comp. Physiol.* **41**, 313, 1953.
- [19] Niemierko S. - *Acta Biol. Exper.* **15**, 101, 1950.
- [20] Niemierko S., *IV Intern. Congress of Biochem. Vienna 1958, Symposium 12, Biochemistry of Insects* (L. Levenbook, ed.) p. 179, Pergamon Press, London, 1959.
- [21] Niemierko S. & Niemierko W. - *Nature* **166**, 268, 1950.
- [22] Niemierko S. & Niemierko W., *IV Intern. Congress of Biochem., Vienna 1958*. Abstr. Comm. p. 151, 12-19. Pergamon Press, 1960.
- [23] Niemierko W., *Colloque Intern. sur les Acides Ribonucleiques et les Polyphosphates*, p. 615, Strasbourg, 1961.
- [24] Niemierko W. & Cepelewicz S. - *Acta Biol. Exper.* **15**, 57, 1950.
- [25] Niemierko W. & Wojtczak L. - *Acta Biol. Exper.* **15**, 79, 1950.
- [26] Parck H. D. & Buck J. - *J. Insect. Physiol.* **4**, 220, 1960.
- [27] Price G. - *Biochem. J.* **86**, 372, 1963.
- [28] Ray J. W. & Heslop J. P. - *Biochem. J.* **87**, 39, 1963.
- [29] Rosenberg H., Ennor A. H. & Morrison J. F. - *Biochem. J.* **63**, 153, 1956.
- [30] Slater E. C. - *Biochem. J.* **53**, 157, 1953.
- [31] Slater E. C. & Holton F. A. - *Biochem. J.* **55**, 530, 1953.
- [32] Wilhelm R. C., Schneiderman H. A. & Daniel L. J. - *J. Insect. Physiol.* **7**, 273, 1961.
- [33] Winteringham F. P. W. - *Biochem. J.* **75**, 38, 1960.
- [34] Wojtczak L. - *Acta Biol. Exper.* **15**, 223, 1952.
- [35] Wyatt G. R., *IV Intern. Congress of Biochem. Vienna 1958, Symposium 12, Biochemistry of Insects* (L. Levenbook, ed.) p. 161, Pergamon Press, London 1959.
- [36] Wyatt G. R., in *The Control Mechanism in Respiration and Fermentation* (B. Wright, ed.) p. 179. The Ronald Press Comp., 1963.
- [37] Wyatt G. R. & Kalf G. F. - *J. Gen. Physiol.* **40**, 833, 1957.
- [38] Załuska H. & Niemierko S., *V Intern. Congress of Biochem. Moscow 1961*, Abstr. Comm. p. 201, 6-17. Pergamon Press 1963.
- [39] Zebe E. & McShan W. H. - *J. Gen. Physiol.* **40**, 779, 1957.

WPLYW ANAEROBIOZY NA ZACHOWANIE SIĘ NIEKTÓRYCH ZWIĄZKÓW FOSFOROWYCH I WĘGLOWODANÓW U MOŁA WOSKOWEGO

Streszczenie

1. Badania prowadzono na różnych stadiach rozwojowych, głównie na gąsienicach, *Galleria mellonella* umieszczonych w atmosferze azotu w 30° na różny okres czasu, aż do 13 godzin. W warunkach beztlenowych wzrasta zawartość ortofosforanu, która z około 4 μ mol może dochodzić do 15 μ mol na 1 g. masy ciała. Równocześnie ilość niektórych związków fosforowych — przede wszystkim ATP i fosfoargininy — zmniejsza się, AMP wzrasta, ADP zaś nie ulega zmianie. W czasie anaerobiozy znika z ciała znaczna część glikogenu i trehalozy. Ilość glikogenu maleje już w pierwszych godzinach anoksji z około 200 do 80 mg%; ta niewielka ilość nie ulega jednak dalszemu zmniejszeniu. Zawartość trehalozy zaczyna spadać dopiero w nieco późniejszym okresie anoksji. Spadek ten po dłuższym czasie doprowadza do zmniejszenia się zawartości trehalozy z 500 do 200 mg%. Nie zaobserwowano przyrostu glicerofosforanu i cukrów prostych. Ilość powstających w warunkach beztlenowych kwasu mlekowego i pirogronowego oraz glicerolu nie pokrywa ilości znikających węglowodanów.

2. Po przeniesieniu gąsienic z warunków beztlenowych do powietrza obserwuje się zmniejszenie ilości nagromadzonych poprzednio produktów oraz odbudowę substancji, które uległy rozpadowi. Zużycie tlenu przez gąsienice w początkowym okresie „wypoczynku“ w powietrzu jest znacznie mniejsze niż w normie i w przypadku 3 godzinnej anaerobiozy dopiero po około 2 godz. pobytu w powietrzu powraca do stanu wyjściowego.

3. Podjęto próbę interpretacji niektórych procesów występujących w czasie braku tlenu w oparciu o opisane przez de Duve'a zjawisko rozpadu lizosomów pod wpływem anoksji. Wysłunięto ponadto przypuszczenie, że obniżone oddychanie gąsienic w początkowych okresach powrotu do normy może być, częściowo przynajmniej, uzależnione od nagromadzenia się w ciele ortofosforanu i AMP i równoczesnego zmniejszenia się ilości ATP.

Received 15 February 1964.

RECENZJE KSIĄŻEK

PHENYLKETONURIA (F. L. L y m a n, ed.). Charles C. Thomas, Publ., Springfield (Ill.) 1963; str. 318, cena \$ 12.75.

W lipcu 1959 odbyła się w Portland (Maine) I Międzynarodowa konferencja "On Mental Retardation", która dała uczestnikom sposobność do bliższego zetknięcia się z odkrywcą fenyloketonurii, dr Asbjörnem Föllingiem. W ten sposób powstała inicjatywa opracowania tej książki, która łączy w sobie elementy biograficzne z naukowym omówieniem problemu oraz przepisami laboratoryjnymi i obszernym traktatem o diecie leczniczej. Książka więc jest przeznaczona zarówno dla biochemików, jak i klinicystów, pielęgniarek, rodziców chorych dzieci, a zainteresuje również historyka medycyny. Dla klimatu tej książki charakterystycznym jest, że wstęp do niej napisała słynna autorka amerykańska, Pearl S. Buck. Uczuciowy stosunek Redaktora książki do zagadnienia wyraża się i w takim szczególe, że barwa okładki mogłaby służyć jako wzorzec do odczytywania próby diagnostycznej na fenyloketonurię.

W I rozdziale Zygyrida i W. R. Centerwall opowiadają historię odkrycia, tak jak ją usłyszeli z ust dr Föllinga. Następnie W. E. Knox przedstawia występowanie i dziedziczność fenyloketonurii. G. A. Jervis jest autorem rozdziałów o klinicznym obrazie tej choroby oraz o patogenecie zaburzeń w rozwoju umysłowym. Stronę biochemiczną zagadnienia omawia obszernie M. D. Armstrong. O metodach wykrywania i rozpoznawania piszą W. R. Centerwall, Helena K. Berry i L. I. Woolf. Rozdział o leczeniu dzieci dotkniętych tym blokiem metabolicznym napisali H. Bickel i W. Grüter; polega ono głównie na podawaniu (kosztownej zresztą) diety ubogiej w fenyloalaninę. Osobny rozdział, autorstwa F. L. Lymana, podaje wybór przepisów dietetycznych. Szczegółowe metody jakościowe i ilościowe dla oznaczania kwasu fenylopirogronowego i hydroksyfenylooctowego podaje L. I. Woolf. Na zakończenie H. A. Waisman omawia krótko doświadczenia na zwierzętach eksperymentalnych.

Bardzo charakterystyczne dla rozwoju zainteresowania fenyloketonurią i pokrewnymi zjawiskami jest chronologiczne zestawienie piśmiennictwa: w okresie 1908 - 1918, 5 publikacji, 1919 - 1933, 5 publikacji, w latach 1934 - 1945, 49 publikacji, w dziesięcioleciu 1950 - 1959, 372 pozycje, w r. 1960, 54, a w r. 1961 już 112.

Józef Heller

ENZYME CHEMISTRY OF PHENOLIC COMPOUNDS. Proceedings of the Plant Phenolics Group Symposium, Liverpool, April 1962 (J. B. Pridham, ed.). Pergamon Press, Oxford 1963; str. IX + 141, cena 50 s.

Omawiana książka zawiera materiały kolejnej konferencji roboczej „grupy fenoli roślinnych” (porównaj recenzję w Acta Biochim. Polon. 9, zeszyt 2, 1962). Konferencja odbyła się w kwietniu 1962 w Liverpoolu. Redaktorem jest J. B. Prid-

ham, który jest również autorem rozdziału o enzymach hydrolitycznych. Ogółem książka zawiera 13 artykułów. Wstępny, pióra N. A. Burgesa, zawiera krótką charakterystykę enzymów, działających na fenole. O oczyszczaniu i testowaniu roślinnych oksydaz fenolowych traktuje jasno i instruktywnie napisany artykuł D. S. Bendalla i R. P. F. Gregory'ego. Wiele mogą z niego skorzystać także pracujący nad enzymami zwierzęcymi. J. G. Boswell dyskutuje zagadnienie, czy fenolazy pełnią rolę końcowych oksydaz oddechowych i stwierdza, że kwestia ta pozostaje nadal otwarta. J. B. Jepson omawia porównawczo najważniejsze reakcje enzymatyczne wprowadzające grupy hydroksylowe do pierścieni aromatycznych. Następne dwa rozdziały poświęcono ważnym dla świata zwierzęcego chinonom. J. Green omawia występowanie tokoferoli, a F. W. Hemming i R. A. Morton rolę ubichinonu i plasto-chinonu u roślin. A. H. Williams omawia hamujący wpływ fenoli na enzymy, mający duże znaczenie praktyczne.

Wymienione rozdziały charakteryzują wystarczająco tematykę wypełniającą ten interesujący tomik. Ważne dla siebie informacje znajdzie w nim przede wszystkim biochemik interesujący się metabolizmem związków fenolowych u roślin, ale również u zwierząt i drobnoustrojów. Książka zawiera też wiele szczegółów, ważnych dla zrozumienia charakteru niektórych ogniw łańcucha oddechowego. Zainteresować powinna ona również tych, którzy, kierując biochemiczną stroną procesów technologicznych, napotykają w praktyce na trudności związane z obecnością i dużą reaktywnością związków fenolowych.

Józef Heller

F. C. Reubi, *CLEARANCE TESTS IN CLINICAL MEDICINE*. Charles C. Thomas, Publ., Springfield (Ill.) 1963; str. XV + 217, 36 tabel i 26 rysunków, cena \$8.75.

Wprowadzenie testów oczyszczania do fizjologii i fizjopatologii nerek pozwoliło na stworzenie podwalin nowoczesnej nefrologii. Podczas gdy w medycynie doświadczalnej testy oczyszczania różnych substancji znalazły bardzo szerokie zastosowanie, to zdecydowana większość ośrodków klinicznych ogranicza się w badaniach rutynowych do oznaczania testu oczyszczania endogennej kreatyniny, mimo że jest on mało dokładnym wskaźnikiem wielkości filtracji kłębkowej. Testy oczyszczania innych substancji wykonywane są tylko w wyjątkowych przypadkach tak ze względu na trudności techniczne, jak i bezpieczeństwo badanego.

Podręcznik F. C. Reubi'ego poświęcony jest omówieniu zastosowania testów oczyszczania w praktyce klinicznej dla celów diagnostycznych i prognostycznych. Podręcznik składa się z dwóch części. Pierwsza z nich (3 rozdziały) obejmuje rozważania teoretyczne, opis metod oraz interpretację wyników. W części drugiej (8 rozdziałów) omówione są wyniki testów oczyszczania w różnych schorzeniach nerek oraz podane są kryteria, które, zdaniem Autora, pozwalają wykorzystać te wyniki w diagnostyce różnicowej oraz w rozważaniach prognostycznych. Podręcznik napisany rozstał na podstawie własnego materiału i własnych obserwacji Autora. Nie ulega wątpliwości, że Reubi, który zajmuje się od kilkunastu lat testami oczyszczania u ludzi zdrowych i u pacjentów ze schorzeniami nerek, jest jedną z najbardziej powołanych osób do napisania tego rodzaju podręcznika. Mimo pełnego uznania dla doświadczenia Autora, trudno jest zgodzić się z niektórymi tezami i zaleceniami zawartymi w omawianym podręczniku. Autor stoi na stanowisku, że ponieważ test oczyszczania endogennej kreatyniny jest (szczególnie w schorzeniach nerek) złym wskaźnikiem wielkości filtracji kłębkowej, powinien on być

zastąpiony przez test oczyszczania inuliny (lub tiosiarczanu). Ponadto Autor uważa, że wyniki testów oczyszczania inuliny (lub tiosiarczanu) i paraaminohippuranu mają bardzo duże znaczenie diagnostyczne i prognostyczne i dlatego testy te powinny być stosowane jako badanie rutynowe. W wybranych przypadkach Autor zaleca przeprowadzenie testów oczyszczania innych substancji oraz badań pochodnych, jak np. ekstrakcji nerkowej paraaminohippuranu, reabsorpcji glikozy i innych. Dla prześledzenia dynamiki procesu wskazane jest okresowe powtarzanie badań. Technika wszystkich tych badań wymaga cewnikowania moczu. W opinii Autora zabieg ten, o ile wykonany jest w warunkach aseptycznych, nie powoduje żadnych szkodliwych następstw. Opinia ta jest zaskakująca, ponieważ jest zupełnie sprzeczna z poglądami zdecydowanej większości internistów. Z licznych badań różnych autorów wynika, że nawet przy bardzo ściśle przestrzeganej aseptyce i antyseptyce już jednorazowe cewnikowanie moczu, nawet nie połączone z wprowadzaniem do pęcherza jakichkolwiek środków przez cewnik (co jest konieczne w testach oczyszczania), powoduje w 5 do 10% przypadków zakażenie dróg moczowych. Stanowi to groźne niebezpieczeństwo już dla osobników zdrowych, a coś dopiero dla pacjentów ze schorzeniami nerek. Groźba zakażenia dróg moczowych była przyczyną, która spowodowała, że większość internistów wykluczyła z arsenału dodatkowych badań rutynowych testy oczyszczania z wyjątkiem testu endogennej kreatyniny, który nie wymaga cewnikowania pęcherza. Zdziwienie budzi fakt, że Reubi kilku zdaniem kwituje problem cewnikowania moczu i przekreśla badania i opinie wielu bardzo poważnych autorów.

Przypisując główne znaczenie testom oczyszczania inuliny (lub tiosiarczanu) oraz paraaminohippuranu Autor omawia przede wszystkim te próby. Szkoda, że równie wyczerpująco nie zostały omówione inne testy, dla których krytycznego omówienia ten właśnie podręcznik jest jak najbardziej odpowiednim miejscem.

W rozdziale poświęconym interpretacji wyników testów oczyszczania oraz wyliczanej z nich wielkości frakcji filtracyjnej Autor, świadomie zresztą, upraszcza szereg zagadnień. Wydaje się jednak, że Autor zbyt marginesowo traktuje fakt, że w schorzeniach nerek u różnych osobników cierpiących na tę samą sprawę chorobową zmiany strukturalne i czynnościowe nerek mogą mieć różny charakter w zależności od bliżej nieokreślonych właściwości osobniczych lub też od współistnienia zmian dodatkowych (np. niedokrwistość, nadciśnienie, niedomoga krążenia i in.).

Część szczegółowa podręcznika jest bogatym i interesującym zbiorem wyników testów oczyszczania uzyskanych przez Autora u pacjentów z różnymi schorzeniami nerek. Przy omawianiu każdej jednostki chorobowej Autor przeprowadza najpierw rozważania teoretyczne dotyczące zmian czynnościowych nerek, a następnie przedstawia korzyści praktyczne, jakie jego zdaniem uzyskać można przez wnikliwą ocenę wyników testów oczyszczania. Korzyści te dotyczą przede wszystkim rozpoznania różnicowego oraz rokowania w chorobach nerek. Ta część podręcznika wymagałaby obszernego omówienia przekraczającego ramy niniejszej recenzji. Ogólnie biorąc wydaje się, że Autor zbyt entuzjastycznie określa wartość praktyczną testów oczyszczania. W niektórych punktach (np. różnicowanie między ostrym, rozlanym a ogniskowym zapaleniem kłębków nerkowych) można mieć wątpliwości co do zgodności wniosków i wyników przedstawionych przez Autora. Wydaje się również, że w pewnych przypadkach Autor wysunął wnioski na podstawie zbyt małej ilości przypadków (np. podział 18 pacjentów z zespołem nerocycowym na 7 grup o różnym obrazie i przebiegu klinicznym).

W sumie podręcznik F. C. Reubi'ego stanowi obszerny i bogaty materiał bardzo pomocny (szczególnie w konfrontacji z wynikami innych autorów) w ugruntowaniu naszej wiedzy w zakresie fizjopatologii nerek. Zdaniem recenzenta nie spełni

on jednak zadania, jakie chciał mu nadać Autor, to znaczy nie zachęci do rutynowego stosowania testów oczyszczania inuliny i paraaminohippuranu. Napewno większość klinicystów ograniczy swoje badania rutynowe do innych, mniej niebezpiecznych testów, na których także można się oprzeć zarówno w rozpoznawaniu różnicowym chorób nerek, jak i w rokowaniu.

Andrzej Manitius

F. B. Straub. BIOCHIMIJA (Przekład z II wydania węgierskiego z r. 1961). Izdatelstwo Akademii Nauk Węgrii, Budapeszt 1963; str. 716.

Rosyjskie wydanie biochemii Strauba jest, obok niemieckiego, drugim tłumaczeniem tego podręcznika.

Biochemia Strauba jest przeznaczona dla studentów medycyny i biochemii. Każdy z autorów podręczników biochemii stoi przed trudną decyzją co do zakresu, jaki ma nadać podręcznikowi. Powszechnie stwierdzany niezwykle szybki rozwój biochemii sprawia trudności w wyborze materiału, nadającego się do przedstawienia w podręczniku uniwersyteckim. Wydaje się, że Autor omawianego podręcznika trudne to zadanie rozwiązał pomyślnie.

Biochemia Strauba została podzielona na trzy główne części. Autor przyjął tu zasadę od dawna stosowaną, mianowicie część pierwsza, najdłuższa (około 450 stron), obejmuje biochemię ogólną usystematyzowaną według klucza chemicznego, druga część, biochemia szczegółowa (około 200 stron), obejmuje problemy uszeregowane według kryteriów fizjologiczno-biologicznych. Ostatnia część, najkrótsza (około 60 stron), zawiera opis głównych metod i podstawowe wiadomości z chemii fizycznej.

Część I składa się kolejno z następujących 9 rozdziałów: białka, enzymy, energetyka organizmu, aminokwasy, węglowodany, lipidy, kwasy nukleinowe, witaminy, hormony. W rozdziale o białkach umieszczono ustęp omawiający w zarysie nowoczesny mechanizm biosyntezy, podano także wzmiankę o zakaźności kwasu rybonukleinowego mozaiki tytoniowej. Na str. 73 we wzorze gramicydyny S pomyłkowo podano kierunek strzałek. Grupa karboksylowa leucyny tworzy wiązanie peptydowe z aminową grupą fenyloalaniny, dlatego strzałka winna być skierowana w odwrotną stronę; to samo odnosi się do wszystkich innych strzałek tego cyklicznego decapeptydu. W drugim rozdziale zasługuje na uwagę jasny wykład o kinetyce reakcji enzymatycznych. Treść pierwszych dwóch rozdziałów umożliwia Autorowi umieszczenie już w trzecim rozdziale tych wiadomości, które w innych podręcznikach mogą być podane dopiero w późniejszych częściach książki. W rozdziale poświęconym energetyce organizmu znalazły miejsce: rola ATP, glikoliza, utlenianie kwasu pirogrogowego, cykl kwasów trójkarboksylowych i końcowy łańcuch oddechowy, przy czym zasługuje na uwagę, że wiadomości o koenzymach (NAD i CoA) zostały podane nie w oddzielnym rozdziale, ale w bezpośrednim związku z procesami. W ten sposób Autor uniknął opisowego, statycznego sposobu podawania wiadomości o budowie koenzymów. Czytelnik wcześniej zostaje zainteresowany dynamicznym sposobem traktowania biochemii, podczas gdy w dawniejszych podręcznikach poświęcano znaczną część książki na opis substancji o znaczeniu biologicznym. W rozdziale czwartym zamieszczono wiadomości o przemianach aminokwasów, opis ich bowiem podano już w rozdziale pierwszym. Zasługuje na uwagę, że oddzielnie omówiono patologiczną przemianę aminokwasów i ich przemianę w drobnoustrojach. W rozdziale piątym zatytułowanym „Węglowodany w żywym organizmie” dynamiczny

sposób traktowania znowu wysuwa się na pierwszy plan. Sposób ten wymaga jednak od czytelnika większego przygotowania z chemii organicznej, niż to było konieczne z podręcznikami dawniejszymi. Rozdział szósty odnosi się do lipidów. Drogi biosyntezy fosfolipidów zostały tu uwzględnione w sposób nowoczesny. Rozdział ten został zakończony uwagami z dziedziny patologii przemiany tłuszczowej. Następny rozdział zawiera wykład o kwasach nukleinowych, uwzględnia przy tym drogi biosyntezy układu purynowego i pirymidynowego. Pewnego rodzaju powtórzeniem jest ponowne omówienie wolnych nukleotydów już poprzednio opisanych w rozdziale o enzymach. Dwa ostatnie rozdziały pierwszej części, o witaminach i hormonach, mają układ najbardziej przypominający dawny sposób wykładu, chociaż i tu biosynteza została uwzględniona do pewnego stopnia, jak np. przy omawianiu kwasu askorbinowego, lub hormonów sterydowych.

Druga, fizjologiczna część podręcznika składa się z siedmiu rozdziałów, zatytułowanych: biochemia trawienia, krwi, mleka, skurczu mięśniowego, tkanki nerwowej, skóry. W pierwszym rozdziale znalazł miejsce wykład o enzymach trawienych, ich budowie i mechanizmie działania. Jednym z obszerniejszych rozdziałów jest rozdział poświęcony krwi, w którym omówiono hemoglobinę (częściowo także w rozdziale o białkach), mechanizm przenoszenia tlenu, przemianę materii ciałek czerwonych, białka osocza i składniki niebiałkowe. Wszędzie uwzględniono aspekt patologiczny, co w znacznym stopniu ułatwi studentom medycyny trudne powiązanie dyscyplin teoretycznych z klinicznymi. Po krótkim czwartym rozdziale poświęconym biochemii mleka następuje rozdział o skurczu mięśniowym, w którym zasługuje na uwagę dobre powiązanie zagadnień chemicznych i fizjologicznych, przy czym morfologia też znalazła odpowiednie naświetlenie. Dwa ostatnie rozdziały poświęcono systemowi nerwowemu, biochemii widzenia i tkanki łącznej. I tu również uwzględniono mechanizm zmian patologicznych.

Ostatnia część zawiera jasny wykład o podstawowych pojęciach fizykochemicznych, wolnej energii, systemach buforowych i potencjale oksydoredukcyjnym. Podano tu także opis ważniejszych metod stosowanych w biochemii: elektroforezy, miareczkowania potencjometrycznego, metody ultrawirówkowej, ekstrakcji przeciwnoobrotowej, chromatografii bibułowej i wymiennicy jonowych. Najwięcej miejsca poświęcono metodom izotopowym.

Po każdym rozdziale Autor umieścił wykaz głównych pozycji bibliograficznych, często obejmujących najnowsze lata. W skorowidzu przedmiotowym korzystne jest podanie kursywą tej strony, na której podano wzór strukturalny danego związku. Korzystne jest także częste odwoływanie się do innych miejsc tekstu, co znacznie ułatwi studentowi przyswajanie materiału.

Wyżej wymienione cechy książki Strauba skłaniają do twierdzenia, że Autor pomyślnie rozwiązał trudne dydaktyczne zadanie stworzenia nowoczesnego podręcznika biochemii.

Tadeusz Korzybski

COMPREHENSIVE BIOCHEMISTRY. (M. Florkin & E. H. Stoltz, eds.) Vol. 8. Proteins (Part 2) and Nucleic Acids. Elsevier Publishing Co. Amsterdam, London, New York 1963; str. 308, cena Dfl. 40.—, sh. 80, DM 44.50.

W recenzowanym tomie znajdujemy w części pierwszej zwięzłe, ale bardzo przejrzyste, omówienie stanu naszej wiedzy o białkach złożonych oraz o hormonach peptydowych i białkowych; część druga tego tomu jest poświęcona kwasom nukleinowym. Rozdziały części pierwszej opracowali: F. R. N. Gurd (lipoproteiny),

A. Gottschalk (glikoproteiny), E. Boeri (metaloproteiny nieporfirynowe), H. Lehmann i R. G. Huntsman (hemoglobina i mioglobina), J. Roche i R. Michel (hormon tarczycy), H. Rasmussen (hormon przytarczyc), Ch. H. Li (hormony przedniej części przysadki), R. Acher (hormony tylnej części przysadki), W. W. Bromer i O. K. Behrens (insulina i glukagon). Autorami rozdziałów części drugiej są: D. M. Brown i T. L. V. Ulbricht (chemia kwasów nukleinowych), M. H. F. Wilkins (trójwymiarowa konfiguracja cząsteczki DNA), J. D. Coombes (fizyczne właściwości roztworów kwasów nukleinowych) oraz G. R. Wilkinson (spektroskopowe właściwości kwasów nukleinowych). Na końcu każdego rozdziału podano piśmiennictwo. Wyrazem dbałości Autorów o podanie aktualnego stanu wiedzy jest np. dodana w druku uwaga podająca najnowsze, ogłoszone w r. 1962, prace o insulinie i glukagonie, które nie mogły być uwzględnione w tekście. Stosunkowo wysoka cena książki znajduje uzasadnienie w bardzo starannej formie wydania. Recenzowana książka będzie cennym nabytkiem biblioteki każdego biochemika, zwłaszcza wykładowcy.

Włodzimierz Mozółowski

NOBEL. THE MAN AND HIS PRIZES. Edited by Nobel Foundation. Elsevier Publishing Co. Amsterdam, London, New York 1962; str. 690. cena Dfl. 30.—, sh. 60, DM 33.50.

Nagroda Nobla jest jednym z najwyższych zaszczytów, jaki może spotkać ludzi, których zasługi mają znaczenie dla całej ludzkości. Zrozumiałe więc jest zainteresowanie dziejami Fundacji oraz osobami, które spotkał ten zaszczyt. Omawiana książka ma za zadanie dostarczenie odnośnych informacji. Książkę opracowało ośmiu autorów; wszyscy oni są związani z Fundacją Nobla długoletnią w niej działalnością i im należy, w dużej mierze, zawdzięczać to, że Fundacja Nobla jest czymś więcej niż instytucją rozdzielającą nagrody wybitnym uczonym, literatom czy działaczom społecznym; jest ona także czynnikiem mającym wpływ na kształtowanie się nie tylko naukowych, ale także moralnych poglądów ludzkości.

Prezes zespołu dyrektorów Fundacji, Henryk Schück, podaje biografię Alfreda Nobla. Ragnar Sohlman, współpracownik i przyjaciel Nobla, wykonawca jego testamentu, który w trzy i pół roku trwającej walce ugruntował istnienie Fundacji i nadał kierunek jej pracy, pisze dzieje jej powstania. Te dwa rozdziały przedstawiają trudy, zwycięstwa i klęski życia Alfreda Nobla, człowieka, który mimo materialnego powodzenia nie wiele doznał szczęścia, w którym walczyły ze sobą mizantropia i miłość do ludzi, którego idea stworzenia nagród dla ludzi - dobroczyńców ludzkości musiała sobie z trudem torować drogę przez ludzką małość, ciasny nacjonalizm, biurokrację i gąszcz prawnych przepisów. Okres od śmierci Alfreda Nobla w grudniu 1896 r. do wydania statutu będącego prawną podstawą Fundacji w czerwcu 1900 r. — to lata ciężkiej, niekiedy zdawało się beznadziejnej, walki.

O nagrodach z dziedziny literatury pisze Anders Osterling ze Szwedzkiej Akademii; omawia on podstawy decyzji w stosunku do wszystkich laureatów literatury do 1961 roku włącznie; czytelnik polski znajdzie tu ciekawe szczegóły związane z przyznaniem nagrody Henrykowi Sienkiewiczowi, dotyczące propozycji mniejszości Komitetu równoczesnego nagrodzenia Elizy Orzeszkowej; interesujące jest również rozważanie udzielenia nagrody Stefanowi Żeromskiemu jako kontrkandydatowi Władysława Reymonta; niezmiernie interesujące jest omówienie podstaw decyzji

Komitetu w stosunku do poszczególnych osób, będących przecież zespołem najwybitniejszych pisarzy naszego wieku.

Göran Liljestrand z Karolińskiego Medyczno-Chirurgicznego Instytutu opracował dział nagród z fizjologii i medycyny. Na ponad 200 stronach omówiono działalność poszczególnych laureatów, ujmując razem badaczy określonych działów; wyróżniono następujące pola badania: mikrobiologia i serologia; chemoterapia; fototerapia i leczenie gorączką; hormony i witaminy; nowotwory; genetyka i mechanika rozwojowa; trawienie, krążenie krwi, oddychanie, wydalanie; chemiczna budowa ciała i przemiana pośrednia; układ nerwowy i narządy zmysłowe. Rozdział ten przedstawia w związku z pracą Komitetu dzieje fizjologii i medycyny w pierwszej połowie naszego wieku. Dowiadujemy się tu także o sprawach, które są potwierdzeniem moralnego znaczenia nagród Nobla: ciężkie kłopoty, których doznał Domagk, gdy w 1939 r. przyznano mu nagrodę, były spowodowane wrogiem nastawieniem Hitlera wobec Fundacji za przyznanie nagrody pokoju Ossietzky'emu; niezależność decyzji Komitetów Fundacji i uczciwość w ocenie wartości poszczególnych laureatów nie zawsze wychodziły na dobre nagrodzonym.

Arne Westgren z Królewskiej Akademii Nauk pisze o nagrodach z chemii, obejmujących zakres od budowy atomu i radioaktywności przez termodynamikę chemiczną, chemię nieorganiczną i organiczną, aż do biochemii. Gwałtowny rozwój tych nauk w dwudziestym wieku z jednej strony ułatwiał pracę Komitetowi, któremu nie brakło kandydatów, z drugiej zaś utrudniał wybór jednego z wielu; i w tym rozdziale, jak we wszystkich innych, dostrzeże czytelnik wielki trud Komitetów przyznających nagrody; kierowanie się wyłącznie istotnymi rzeczowymi względami było nakazem, od którego Komitety starały się nie odstępować ani o krok.

Manne Siegbahn, z Królewskiej Akademii Nauk, laureat Nobla z fizyki z 1924 roku, przedstawiając pracę nad przyznaniem nagród z fizyki w latach 1901 - 1961, kreśli właściwie dzieje tej nauki w okresie jej najbardziej intensywnego rozwoju. W końcu XIX wieku wielu fizyków uważało, że wszystko co najważniejsze zostało już odkryte i zbadane, a dla przyszłych pokoleń pozostaje jedynie uzupełnianie szczegółów; później jednak przychodzą odkrycia i teoretyczne ujęcia, których następstwem jest zupełna rewolucja naszych poglądów w dziedzinie fizyki. Te badania, których znaczenia dla ludzkości, znaczenia dobroczynnego, ale i zbrodniczego, nikt dziś nie może zaprzeczyć, nie wydają się słabnąć w swym rozwoju.

O nagrodzie pokoju pisze August Schou z Norweskiego Instytutu Nobla. Nie łatwe zadanie miał, i ma, ten Komitet: przyznanie nagrody osobie (lub instytucji), która przyczyniła się w szczególnej mierze do wzrostu braterstwa między narodami, zniesienia lub zmniejszenia stałych armii i do inicjowania i popierania pokojowych kongresów. W bardzo burzliwej pod względem politycznym pierwszej połowie naszego wieku nie brak było poczynąjących cele humanitarne i pokojowe. Piękny początek nagród Nobla to tak bardzo zasłużone, choć bardzo spóźnione, uznanie dla Henryka Dunant, twórcy Czerwonego Krzyża. W dalszych latach znajdujemy liczne nazwiska nagrodzonych działaczy, z których nie wszystkim dziś przyznalibyśmy to wyróżnienie, ale pamiętać musimy, że przewidywanie skutków działań politycznych nie jest rzeczą łatwą. Autor tego rozdziału zwraca uwagę na szczególne trudności podjęcia decyzji; uważa jednak, że brak jednego tylko nazwiska wśród nagrodzonych nie znajduje usprawiedliwienia, a mianowicie Gandhi'ego. Wśród laureatów pokoju znajdujemy ludzi o bardzo rozmaitych światopoglądach i różnych rodzajach działalności; wspólnym było jedno: dążenie do utrzymania i podniesienia godności człowieka.

Nils K. Ståhle, dyrektor Fundacji do 1948 roku, pisze o finansach i administracji

Fundacji Nobla. Zasługuje na przytoczenie fakt, że nigdy kosztu administracji nie przekroczyły 5% dochodów.

Pełny tekst statutów Fundacji i jej Instytutów oraz wykaz wszystkich osób i instytucji nagrodzonych w latach 1901-1961 kończy tę bardzo ciekawą książkę. Indeks obejmujący ponad tysiąc nazwisk wymienionych w tym dziele pozwala na pełne wykorzystanie zawartych w nim wiadomości.

Włodzimierz Mozołowski

PHYSICAL PROPERTIES OF THE STEROID HORMONES (Lewis L. Engle, ed.). International Series of Monographs on Pure and Applied Biology, vol. 3. Pergamon Press, Oxford, London, New York, Paris, 1963; str. 488, cena £ 7.

Zbiorowy podręcznik wydany pod redakcją Prof. Engela jest niezbędny dla wszystkich pracowni biochemicznych, a w szczególności dla biochemików pracujących nad związkami steroidowymi. Zgromadzone zostały w nim dane fizyczne naturalnych i półsyntetycznych związków steroidowych, niezbędne przy pracach nad ich wyosobnieniem i analizą.

Dział współczynników rozdzielczych, ważny dla wyosobnienia związków steroidowych, został opracowany przez Prof. L. Engela i dr P. Cartera. Po krótkim wstępie teoretycznym i po zarysie techniki badań, podane zostały w układzie tabelarnym współczynniki rozdzielcze najważniejszych steroidów w różnych układach rozpuszczalników.

Dział chromatografii steroidów opracowany został przez dr R. Nehera. Również po krótkim wstępie teoretycznym zamieszczono zarys techniki chromatografii steroidów i uwidoczniania ich metodami barwnymi. Zamieszczono również tabelę danych chromatograficznych najważniejszych steroidów przy różnych układach rozpuszczalników.

Największy dział widm absorpcyjnych steroidów w ultrafiolecie opracowali dr J. P. Dusza, M. Heller i S. Bernstein. Po bardzo krótkim wstępie technicznym i teoretycznym podano wiele tabel widm absorpcyjnych w ultrafiolecie, bardzo wielu naturalnych i półsyntetycznych związków steroidowych. Bez danych tych żadna pracownia naukowa nie może obecnie pracować.

Widma fluorescencyjne związków steroidowych opracował J. W. Goldzieher podając tabelę tych widm dla najważniejszych związków steroidowych. Dział ten zresztą w skali światowej nie jest jeszcze dostatecznie opracowany.

Ostatni dział podręcznika, „Widma absorpcyjne steroidów w stężonym kwasie siarkowym“, opracowany został przez dr L. L. Smitha i B. Bernsteina. Zgromadzono tam również w układzie tabelarnym i wykresach widma najważniejszych związków steroidowych. Absorpcja steroidów rozpuszczonych w kwasie siarkowym często daje bardzo charakterystyczne parametry. W podręczniku brak opracowania widm steroidów w podczerwieni. Prawdopodobnie zostanie uwzględnione to w następnych wydaniach, bo nagromadzony materiał nie jest jeszcze wystarczający do syntetycznego ujęcia tego zagadnienia.

Na końcu podręcznika znajduje się indeks omawianych związków steroidowych, pozwalający na szybkie znalezienie opisu ich własności fizycznych.

Janusz Supniewski