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ISOLATION AND PROPERTIES OF HUMAN SERUM AMYLASE

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Although amylases belong to the better known enzymes and human serum amylase has been a subject of interest for nearly a hundred years, much is still to be learned about this enzyme.

At first studies were directed toward elucidating the origin of human serum amylase, and it is only since about 1950 that systematic attempts have been made to isolate this enzyme and to study some of its properties. Optimum activity at pH 7 has been found [13], and the activation by chloride ions. McGeachin & Lewis [17] observed that elimination of chlorides from the albumin fraction by dialysis against distilled water causes a loss of amylolytic activity which can be restored by re-addition of sodium chloride. Divergent results were obtained in studies of electrophoretic mobility. McGeachin & Lewis [17] employing veronal buffer, pH 8.6, found the largest amount of amylase in albumins, while the rest of it was about equally distributed among the other protein fractions. Kunkel & Ebenhard (cit. after [17]), who employed a plastic powder (Geon) support for electrophoresis, also found the greater part of amylase in the albumin fraction, but only traces in α- and β-globulins, and no activity at all in γ-globulins. On the other hand, Baker & Pellegrini [1] found amylase mainly in the γ-globulin fraction.

The isolated amylase preparations differed somewhat in respect to activity, but it never exceeded 0.1% of the activity of tissue amylase [8, 13].

In this study an attempt was made at isolating amylase of a greater degree of purity, by selective adsorption. The method of adsorption on substrates at temperatures slightly above 0° has been employed for isolating α-amylase from malt, *Pseudomonas saccharofilae*, and *Clostridium acetobutylicum* with good results. For instance Schwimmer & Balls [22], Thayer [23] and Markovitz *et al.* [16] by passing solutions containing amylase through a starch-celite column achieved considerable concentration and purification of the enzyme.

Of the reagents used for precipitating the enzymes from solutions, only acetone gave good results; acetone was also employed by Yoshida & Yamasaki [24] for crystallizing α-amylase from *Bacillus subtilis*.

EXPERIMENTAL

Materials

Human plasma was obtained by centrifuging preserved citrated blood. One liter of plasma was derived from the blood of about 50 donors.

Starch for adsorption was prepared from commercial potato starch by repeated washing with water. The 5- and 10-minute fractions were used, i.e. fractions sedimenting in water within 5 or 10 min. 3,5-Dinitrosalicylic acid was prepared by the method of Hübner [10]. Ethylcellulose was prepared according to Flodin & Kupke [7]. Cellulose-acetate strips were a product of the Oxo Ltd. Comp. (London).

Methods

Amylase activity was estimated by two methods. (1) The iodine method of Hanes & Cattle [9] was modified by reducing the volume to 10 ml. and eliminating inhibition with hydrochloric acid, since it was found that addition of iodine itself is sufficient to stop the activity of amylase. The amount of amylase which under the conditions used decreased by 0.001 the extinction at 575 mμ (10 mm. cuvet) of a 0.1% solution of starch was taken as the amylase unit. This amount corresponded to 26 μg. of decomposed starch per liter of solution. (2) The reduction method with 3,5-dinitrosalicylic acid was performed according to Noelting & Bernfeld [19] and the activity was expressed in mg. of maltose released from starch during 3 min. at 20°.

Protein was estimated with the Folin-Ciocalteu reagent [11], and spectrophotometrically at 280 mμ using SF4 apparatus. Nitrogen was determined by the micro-Kjeldahl method.

Column electrophoresis on ethylcellulose was carried out in the apparatus illustrated in Fig. 1. A column (1 × 150 cm.) was filled using a technique similar to the one described by Porath [20]. Onto the column 1.5 to 2 ml. of amylase solution (2 mg./ml.) was applied. A potassium phosphate buffer, pH 7.7 and ionic strength 0.05 was used for electrophoresis. Silver electrodes were immersed in saturated KCl solution. A current of 3 mA and 560 V was applied. The temperature in the column was 15 - 16°, and the time of electrophoresis was 90 hr. Amylase was eluted with 0.01 N-ammonium hydroxide, and fractions of 1.5 ml. were collected at 10-min. intervals. Each fraction was examined by the iodine method for amylase activity, and spectrophotometrically for protein content.

Electrophoresis on cellulose-acetate strips was performed by the technique described by Kohn [12], and on Whatman no. 3 MM paper according to McGeachin & Lewis [17].

Chromatography on starch-celite column (4 : 1) was carried out according to Thayer [23] except that distilled water instead of buffer solution was used for

the washing, and 0.01 N-ammonium hydroxide instead of a gradient of starch solution for elution. The columns were 2.7 cm. in diameter and were filled with a mixture of starch-celite (5-min. fraction) to a height of 5 cm. After applying amylase, the column was washed with 50 ml. of water adjusted to pH 8.4 with ammonium

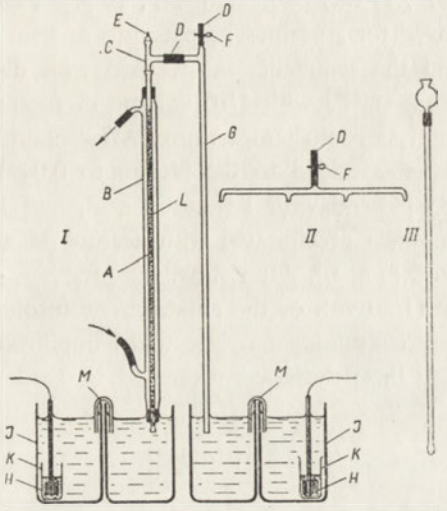

Fig. 1. (*I*), Apparatus for column electrophoresis; *A*, column; *B*, cooler; *C*, connecting piece; *D*, rubber tubes; *E*, stopcork; *F*, clamps; *G*, connecting tube to the vessel; *H*, electrodes; *J*, vessels; *K*, beakers; *L*, ethylcellulose; *M*, bridges. (*II*), Siphon for regulating the level of fluid in the vessels. (*III*), Funnel for filling the column

hydroxide. Elution was done at room temperature, and 3-ml. fractions were collected at a rate of about 12 ml. per hour. The activity of each fraction was estimated by the iodine method, and protein content with the Folin-Ciocalteu reagent.

Stability of amylase at pH ranging from 3 to 5 was studied in 0.5 M-acetate buffers, from pH 5.5 to pH 8 in 0.1 M-phosphate buffers, and from pH 8.5 to pH 11.0 in 0.1 M-glycine - NaOH buffers. Solutions of amylase (50 μg. per ml.) were mixed with equal volumes of 0.2 M buffer solutions and left at room temperature. After 24 and 48 hr. pH was adjusted to 7 with phosphate buffer and the activity was examined by the iodine method.

Estimations of the energy of activation were made by determining the degree of decomposition of the substrate by the iodine method at temperatures from 0° to 45°.

The Michaelis constant was determined by measuring the kinetics of hydrolysis by the reduction method with 3,5-dinitrosalicylic acid in the range of starch concentrations from 0.05 to 5%, and by the iodine method in the range from 0.001 to 0.5%.

RESULTS

Isolation

Citrated plasma (pH 7.1) was cooled to +2°, 10 g. starch (10-min. fraction) per liter was added and mixed for 30 min. The plasma was then removed by siphoning and centrifugation, and the sediment was washed with distilled water at 0 - 2°. Amylase was eluted from starch with 1/10 volume of plasma of 0.01 N-NH_4OH, with shaking for 30 min. at room temperature. After filtering off of the insoluble matter, calcium acetate was added to the filtrate to 0.04 M concentration. The solution was adjusted to pH 9.5 with 1 N-acetic acid, and left in the refrigerator for 2 days. Amylase was then precipitated with acetone at a final concentration of 60%, at pH from 8 to 9.5, at 0°. After 2 hr. the precipitate was centrifuged at 0° and dissolved in water (1/20 vol. of the eluate). The insoluble sediment was discarded, and the solution containing amylase was lyophilized. The recovery of the activity was about 10%; the details are presented in Table 1.

Table 1

Isolation of amylase by selective adsorption

Citrate blood plasma was used. Amylase activity was estimated by the iodine method. Average values from 6 samples of 2000 ml. each are given

Material	N (mg.)	Activity		Purification
		(unit/mg.N)	(%)	
Plasma	17500	500	100	
Plasma after adsorption	16300	80	15	
Washings I	99	2700	3.2	
Washings II	14	9500	1.6	
Washings III	3	15500	0.6	
Eluate	6.3	500000	45	1000
Acetone precipitate	1.5	3700000	10	7000

The amylase preparation obtained by selective adsorption and containing 5×10^6 to 1×10^7 units was further purified by starch-celite column chromatography; it emerged as a peak of activity (Fig. 2b) of about 0.7×10^6 units per mg. with a recovery of 40 - 60%. The active eluates, containing 5×10^6 units, after rechromatography showed an activity of 1.2×10^6 to 1.5×10^6 units per mg. of protein with a recovery of about 60% (Fig. 2c). A similar degree of purification was achieved by chromatographing twice the preparation obtained by ethanol fractionation [8].

The active eluate fractions from the re-chromatography were combined and precipitated with 60% acetone. The preparation showed activity of about 2×10^6 units per mg., i.e. 66 mg. of maltose per mg. of enzyme. The recovery of the acti-

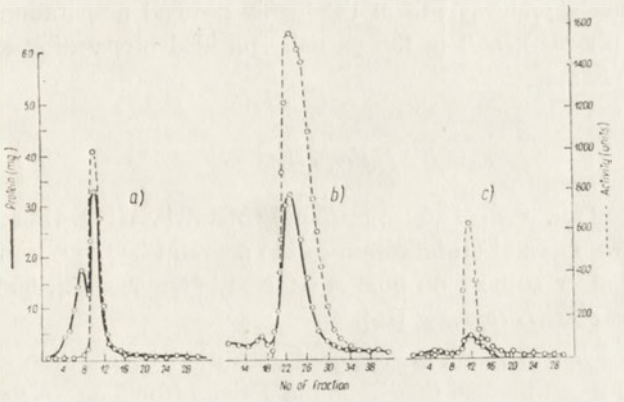

Fig. 2. Chromatography on starch-celite column of plasma amylase preparations obtained by: (*a*), ethanol fractionation; (*b*), selective adsorption; (*c*), rechromatography of active eluates

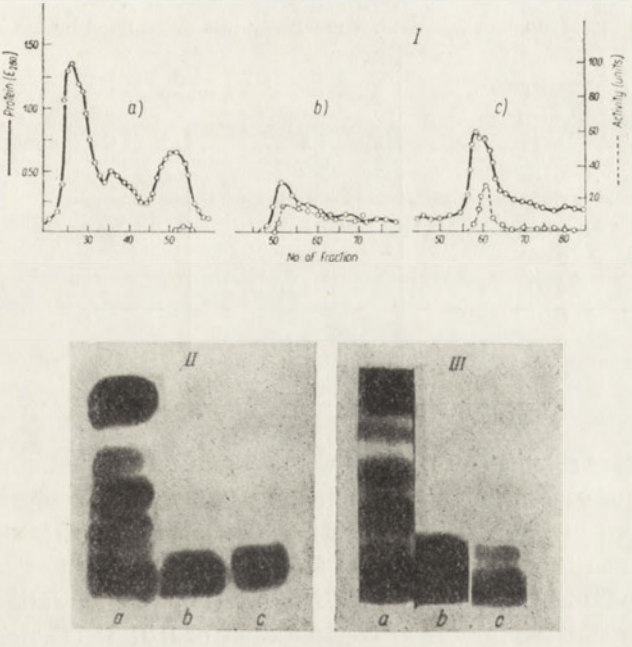

Fig. 3. Electrophoresis on (*I*), ethylcellulose column; (*II*), cellulose-acetate strip; (*III*), Whatman no. 3 MM paper. (*a*), Blood serum; (*b*), preparation of amylase after selective adsorption; (*c*), purified preparation (activity: 66 mg. maltose/mg.). Details see Methods

vity was very low; from 10 liters of plasma, 0.5 - 1 mg. of preparation was obtained containing only 2 — 5% of the initial activity.

Electrophoresis on a column of ethylcellulose, on cellulose-acetate strips, and on paper (Fig. 3) showed predominance of the protein fraction with a mobility of slowly-moving globulins, both in the highly purified preparation (with activity 2×10^6 units per mg.), and in the partially purified preparation after direct adsorption from plasma.

Properties

The ultraviolet spectrum of aqueous solution of amylase (Fig. 4) showed maximum absorption at 278 mμ and an inflection of the curve at 290 mμ, in agreement with the findings of other authors on human pancreatic amylase [2], human saliva [3], and *Pseudomonas saccharophilae* [16].

The pure amylase preparation required activation by chloride ions. From the data in Table 2 it can be seen that optimum activity (for 5 μg. of the enzyme) was achieved at the concentration of 0.05 M-NaCl.

Table 2

Effect of chloride ions on the activity of serum amylase

Per sample 0.5 μg. of amylase was used. The activity was determined by the iodine method.

NaCl concn. (M)	Activity	
	(units)	(% of the optimum)
0	20	5
0.0005	270	66
0.001	295	72
0.005	310	75
0.01	360	88
0.05	410	100
0.1	410	100

Dilute aqueous solutions of amylase were very unstable. Kept at room temperature, solutions of concentration 1 μg./ml. completely lost their activity after 5 hr.; at concentrations of 10 μg./ml. inactivation took place more slowly, the activity of the preparation being diminished by 50% after 24 hr. Bivalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) had a stabilizing effect. Amylase in concentration of 5 μg./ml. in the presence of these cations in concentration of 0.01 M (Table 3) lost about 30% of its activity after 5 days, and 30 - 50% of activity was still present after 2 months. Ions of Na^+ and K^+ had a much weaker effect. Heavy metals inactivated amylase: Zn^{2+} markedly, and Cu^{2+} completely, within a few hours.

Dialysis during 40 hr. against distilled water at 4° caused irreversible loss of 90% of activity. Sutra [21] reported similar observations on human salivary amylase.

The activity of amylase kept for 48 hr. in a medium of pH 6.5 - 11.5 was not changed; below 6.5 amylase was quickly inactivated (Fig. 5). Compared with the

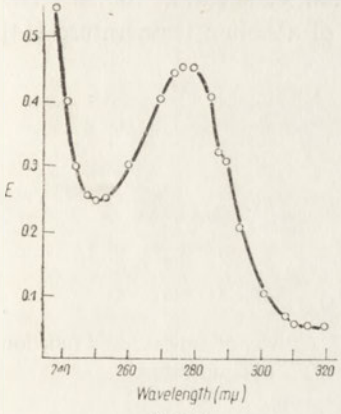

Fig. 4

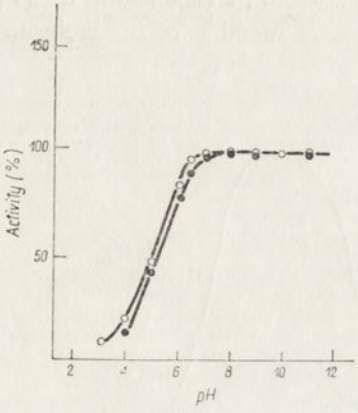

Fig. 5

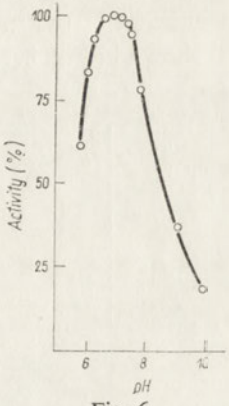

Fig. 6

Fig. 4. Absorption spectrum of plasma amylase (0.5 mg. amylase/ml. water).
Fig. 5. Stability of amylase in relation to pH. Solutions of amylase were kept stored at different pH for (○), 24 hr; (●), 48 hr.
Fig. 6. Activity of amylase as a function of pH

values given by Bernfeld *et al.* [3, 2] for human salivary or pancreatic amylase (Table 5), plasma amylase appears to show stability at a somewhat different range of pH. This was not due to differences in the experimental procedure, since incubation of plasma amylase in the conditions used by Bernfeld *et al.* [3] gave identical results with those shown in Fig. 5. Optimum activity of amylase was found at

Table 3

Effect of cations on the stability of amylase

To a solution of amylase (5 mg./ml.) appropriate chlorides were added. After indicated time at room temperature, the activity of amylase was determined by the iodine method and expressed in amylase units.

Cation (0.01 M)	Time of storing			
	2 hr.	24 hr.	5 days	2 months
Na^+	370	240	30	10
K^+	300	210	90	10
Ca^{2+}	465	370	260	—
Mg^{2+}	445	370	320	255
Mn^{2+}	345	280	370	165
Zn^{2+}	170	30	0	0
Cu^{2+}	50	0	0	0

pH 7 ± 0.3; outside of this range, the activity quickly decreased; the curve is not quite symmetrical as shown in Fig. 6. Optimum temperature was 40°; at 65° amylase was completely inactive (Fig. 7).

The activation energy, calculated by means of the equation of Arrhenius, was 11 600 cal. in the range 0 - 40°, 12 200 cal. at 0 - 30°, and 9 500 cal. at 10 - 30°. The logarithm of the rate as a function of the reciprocal of absolute temperature [14] is shown in Fig. 8.

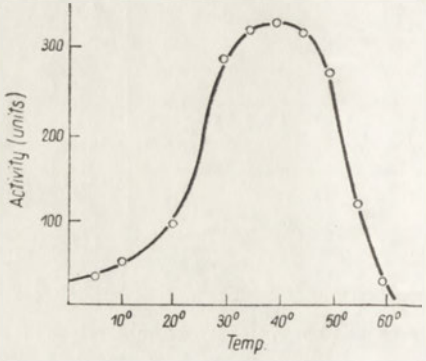

Fig. 7. Activity of amylase as a function of temperature

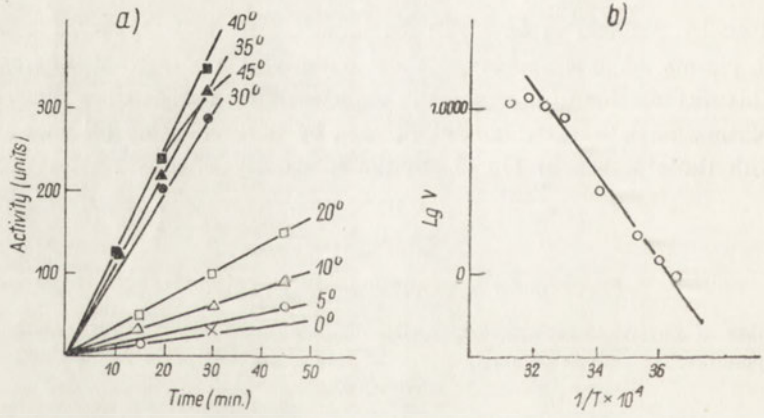

Fig. 8. Arrhenius function. (*a*), Course of starch hydrolysis in time; (*b*), logarithm of the rate as a function of the reciprocal of absolute temperature

Amylase in the dilution 1 μg./ml. showed increasing activity in proportion to increasing concentration of substrate, reaching a maximum at the concentration of 0.05% of starch. With greater concentrations activity diminished (Fig. 9).

The Michaelis constant calculated by the graphic method of Lineweaver & Burk [15] in the range of starch concentrations from 0.05 to 0.5% was 0.65 g. of starch

per liter. The same value was found for preparations purified by ethanol fractionation (2×10^4 units/mg.), obtained after selective adsorption (3×10^5 units/mg.), and purified by repeated chromatography (2×10^6 units/mg.). The rate of reaction

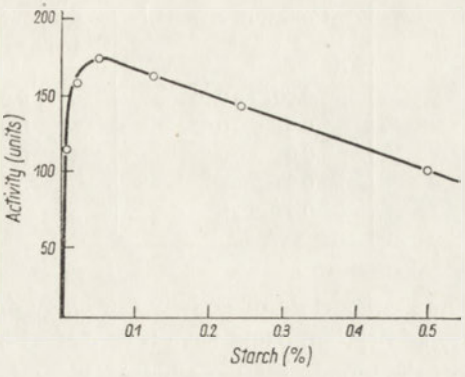

Fig. 9. Activity of plasma amylase in relation to concentration of substrate (1 μg. amylase/ml.)

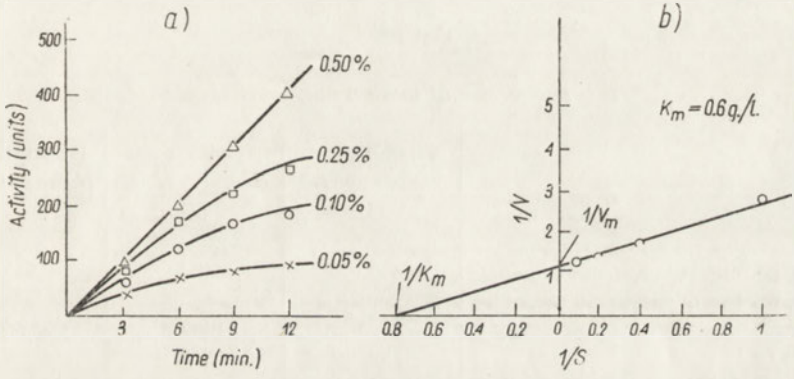

Fig. 10. Michaelis constant for plasma amylase. (*a*), Course of starch hydrolysis in time; (*b*), graphic determination of the Michaelis constant (starch concentrations 0.05 - 0.5%, time of hydrolysis 3 min.)

(V) was determined graphically from the initial phase of starch digestion by amylase. The values obtained at different concentrations of the substrate were plotted on the curve as $1/V$ against $1/S$ (Fig. 10). The values of Michaelis constants at different concentrations of the substrate are collected in Table 4.

DISCUSSION

The use of adsorption on potato starch permitted the separation of amylase from the plasma proteins by a method which is fairly rapid and simple, and less expensive than the previously described method of ethanol fractionation [8].

Table 4

Michaelis constant at different substrate concentrations

Amylase preparation obtained by ethanol fractionation was used.

Concn. of starch (%)	K_m (g./l.)	
	Iodine method	Reduction method
0.001 — 0.01	0.06	
0.01 — 0.05	0.09	
0.05 — 0.5	0.7	0.65
0.05 — 5		0.9
0.5 — 5		1.2

The marked loss of activity especially during the first stage of isolation by selective adsorption, could have been caused by sodium citrate used for the preservation of the blood. Plasma amylase, like all other α-amylases [6, 25] probably contains calcium, and its loss may lead to diminished stability of the enzyme [6].

Table 5

Properties of human amylases

	Crystalline salivary amylase [3]	Crystalline pancreatic amylase [2]	Preparation of plasma amylase
Activity, per mg. N in mg. of maltose	6400	6400	943
Activity, per mg. of enzyme in mg. of maltose	1000	1000	66
Nitrogen (%)	15.8	15.8	7
Phosphorus (%)	0.01	0.01	
Optimum pH	6.9	6.9	6.7 - 7.3
Optimum temperature	40°[a]		40°
Activation by Cl^-	+	+	+
Stability at pH	4.5 - 11	4.5 - 11	6.5 - 11.5
Solubility	0.3% (2° pH 8.7)	0.5% (pH 8)	2% or more
Electrophoretic mobility	3.75×10^{-5} [b]	3.75×10^{-5} [b]	γ-globulin[c]
Absorption spectrum maximum (mμ)	280	280	278
breaking point (mμ)	292	292	290
Michaelis constant; starch concentrations: 0.05 - 0.5%	0.6 ± 0.2 g./l.[d]		0.65 g./l.
Activation energy, 10° - 30° (cal.)	16 500[e]	16 500[e]	9500

[a] Values according to Sutra [21]. [b] Free electrophoresis. [c] Zonal electrophoresis. [d] Values found by Fischer for crystalline amylase from human saliva and pig pancreas (cit. after [21]). [e] Values cited by Fischer & Haselbach [5] for human amylase (organ not indicated), and by Meyer [18] for human salivary and pancreatic amylase (temperature range not indicated).

After double purification by column chromatography, preparations were obtained with an activity of about 66 mg. of maltose per mg. of preparation, i.e. 6% of the activity of salivary or pancreatic amylase. The preparation was purified about 25 000-fold in relation to the activity of plasma, and appears to be about 60 times as pure as the amylase preparation described by Kugelmass [13] even though the nitrogen content (Table 5) in our preparation indicates a marked degree of contamination by a non-protein component.

The final preparation of amylase is a colourless powder soluble in water, activated by chloride ions and showing optimum activity at pH 7 $\pm$ 0.3.

The electrophoretic patterns of the preparation with the activity of about 2×10^6 units/mg. showed a deformation of the curve in column electrophoresis, and in paper electrophoresis the presence of an inactive protein with the mobility of γ-globulin (Fig. 3), indicating the non-homogeneity of the preparation. However, this might have been due to partial denaturation of amylase during electrophoresis. The loss of about 80% of activity during column electrophoresis supports this supposition. Other experiments to evaluate the homogeneity of the preparation could not be performed because the available amount of highly purified material was very small.

Although the enzyme was not obtained in crystalline form, it seemed admissible to characterize the preparation obtained by the method described herein. Marked differences were not observed between preparations of various degree of purification in respect to the Michaelis constants, activation energy, effect of pH on activity, and absorption spectra. The only differences observed viz. in the stability at different pH and in susceptibility to heat inactivation, were very small. They were probably due to the elimination of some impurities having a protective effect on the enzyme.

From the data in Table 5 it can be seen that only some of the properties of human plasma amylase are different from those of human salivary or pancreatic amylase. The specific activity of plasma amylase is much lower, amounting to about 15% of the activity of salivary or pancreatic amylase calculated per mg. of nitrogen; the range of stability on the acid side is smaller; the solubility is greater, and activation energy is different. On the other hand, its sensitivity is equal to that of salivary amylase [28], i.e. it easily loses activity in the presence of certain metals such as copper or zinc, in dilute solutions, and in the presence of metal-binding factors. This latter sensitivity is probably due to the small amount of calcium strongly bound with the enzyme. Amylase from *Bacillus subtilis* and pancreatic amylase from pigs exhibit similar sensitivity, elimination of calcium narrowing the range of their stability on the alkaline side from pH 10 to about 6 [6].

The electrophoretic mobility of plasma amylase is identical with that of salivary and pancreatic amylase, and it has a similar absorption spectrum; it is also activated by chloride ions. The optimum temperature is the same, but plasma amylase is inactive at 65°.

The study of the Michaelis constant (K_m) showed that human plasma amylase has greater affinity to the substrate at low concentrations than at higher ones (Table 4). At starch concentrations in the range 0.05 - 0.5% K_m was 0.65 g./liter, and in the range 0.001 - 0.01% K_m was 0.06 g./liter. It might seem that this difference was due to the method of determining the activity of amylase (iodine method for low concentrations, reduction method for high concentrations). However, this was not the case, as shown by the experiments with concentrations 0.05 - 0.5%, in which K_m determined by each method was the same. In studies on the kinetics of the reaction at low concentrations it was not possible to use the reduction method with 3,5-dinitrosalicylic acid which was employed by Bernfeld & Studer-Pecha [4] for determinations of the Michaelis constant of bacterial and pancreatic amylase. On the other hand, satisfactory results were obtained with the iodine method. The experimental data indicate that excess of substrate inhibits the activity of plasma amylase by diminishing the affinity of the enzyme for the substrate.

The author wishes to express her gratitude to Prof. Dr. K. Zakrzewski for his interest and valuable discussions during the course of this work and to Mrs. Eleonora Grygalun for her valuable technical assistance.

SUMMARY

A method is described for isolating amylase from human plasma by selective adsorption on insoluble potato starch followed by chromatography on starch-celite column and by acetone precipitation. Using this method a 25000-fold purification was obtained. The properties of the preparation were studied and compared with the data on salivary and pancreatic amylase.

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IZOLOWANIE AMYLAZY Z OSOCZA LUDZKIEGO I NIEKTÓRE JEJ WŁAŚCIWOŚCI

Streszczenie

Podano metodę izolowania amylazy z osocza ludzkiego przez selektywną adsorpcję na nierozpuszczalnej skrobii kartoflanej, chromatografię na kolumnie skrobiowo-celitowej i wytrącanie acetonem. Uzyskano preparat o aktywności ok. 66 mg.maltozy/mg. enzymu, to jest oczyszczony 25 000 razy. Zbadano właściwości otrzymanego preparatu i porównano je z właściwościami amylazy śliny i trzustki.

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CHROMATOGRAPHIC SEPARATION ON PHOSPHATE-CELLULOSE OF LIGHT-SENSITIVE FORMS OF CORRINOIDS PRODUCED BY PROPIONIC ACID BACTERIA*

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The ion exchange resins so far have not been widely applied for the separation of corrin compounds. Although they are employed for separating vitamin B_{12} (e.g. Amberlite IRC-50), their role is rather that of adsorbents, like charcoal, than of separating agents. It is only recently that Menke [4, 5, 6] demonstrated the separation of some analogues of vitamin B_{12} on Dowex 50 × 4 resin, and Barker and co-workers [1, 2, 12] employed the same resin for purification of coenzyme forms of several corrinoids.

This limited application of the ion-exchange resins is due to their aromatic character and crossing structure; the charged corrinoids often are not adsorbed at all, or their adsorption is irreversible. The cellulose exchangers, free from these disadvantages, were used for the preparation and analysis of corrinoids by Pawełkiewicz, Walerych, Friedrich & Bernhauer [10].

When the light-sensitive corrinoids, more basic than the cyanide forms, were discovered, it became necessary to find a method of separating them. Carboxymethylcellulose has already been used successfully for the isolation of SB_{12p} [8]. In the present communication a method is presented for separating light-sensitive forms of corrinoids on a column of phosphate-cellulose employing buffers of different concentrations and pH. This method was used for separating the complex mixture of light-sensitive corrinoids obtained from propionic bacteria.

EXPERIMENTAL

Abbreviations. P-cellulose, phosphate-cellulose; ψB_{12}, pseudovitamin B_{12}; FA, the factor A; B_{12p}, cobinamide; y2, phosphocobinamide; B_{12}(COOH), monocarboxylic derivative of vitamin B_{12}; B_{12p}(COOH), carboxylic derivatives of cobinamide.

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The prefix "S" with symbols of the corrin compounds denotes the light-sensitive form, e.g. SB_{12} is the light-sensitive form of vitamin B_{12}.

Standards. Vitamin B_{12}, cobinamide, phosphocobinamide, carboxylic derivatives of cobinamide and of vitamin B_{12} have been isolated from the cultures of *P. shermanii*. Pseudovitamin B_{12} has been isolated from *P. arabinosum*. Factor A was obtained from Prof. Dr. K. Bernhauer.

Isolation of light-sensitive corrinoids from bacterial cells

P. shermanii, *P. petersonii*, *P. freudenreichii* and *P. arabinosum* cells were used as starting material. When necessary, the cells were stored in the frozen state at -10°. All the procedures were performed in a room illuminated with dim-red light.

The organisms collected from 8 - 10 lit. of medium were suspended in 800 ml. of water, adjusted with acetic acid to pH 4.5, and heated to 75°. The suspension was kept at this temperature for 5 min., then quickly cooled to room temperature and centrifuged at 3000 r.p.m. for 30 min. The sediment was re-extracted with 300 ml. of water. The pooled clear aqueous solutions were saturated with solid ammonium sulphate and the corrinoids extracted with *n*-butanol. From butanol they were transferred to water by shaking the butanol phase several times with water. The aqueous solution was desalted by extracting several times with phenol-chloroform mixture (1 : 3, v/v). The pooled phenolic solutions were washed with an equal volume of water, then one volume each of chloroform and *n*-butanol were added, followed by extraction of the corrinoids with small amounts of water. The aqueous extract was washed with chloroform, and traces of the solvents were removed on the water bath at 50° under reduced pressure. The obtained solution of corrins, about 150 - 200 ml., was used for chromatographic separation.

Table 1

Identification by paper electrophoresis of corrinoids from propionic bacteria in chromatographically separated fractions

The chromatography on P-cellulose is presented in Fig. 1.

No. of samples	Fraction	Identified
65 - 70	I	Sy2, SB_{12p}(COOH)
110 - 120	II	SB_{12}, SB_{12}(COOH), $S\psi B_{12}$
130 - 135	III	SFA
150 - 165	IV	not identified
170 - 185	V	SB_{12p}

Separation of corrinoids on a column of P-cellulose

Six g. of P-cellulose, obtained by the method of Peterson & Sober [11], was suspended in 100 ml. of 2 M-acetic acid and transferred to a column of 32 mm. diameter containing a G2 sintered glass plate. When the acid had passed through,

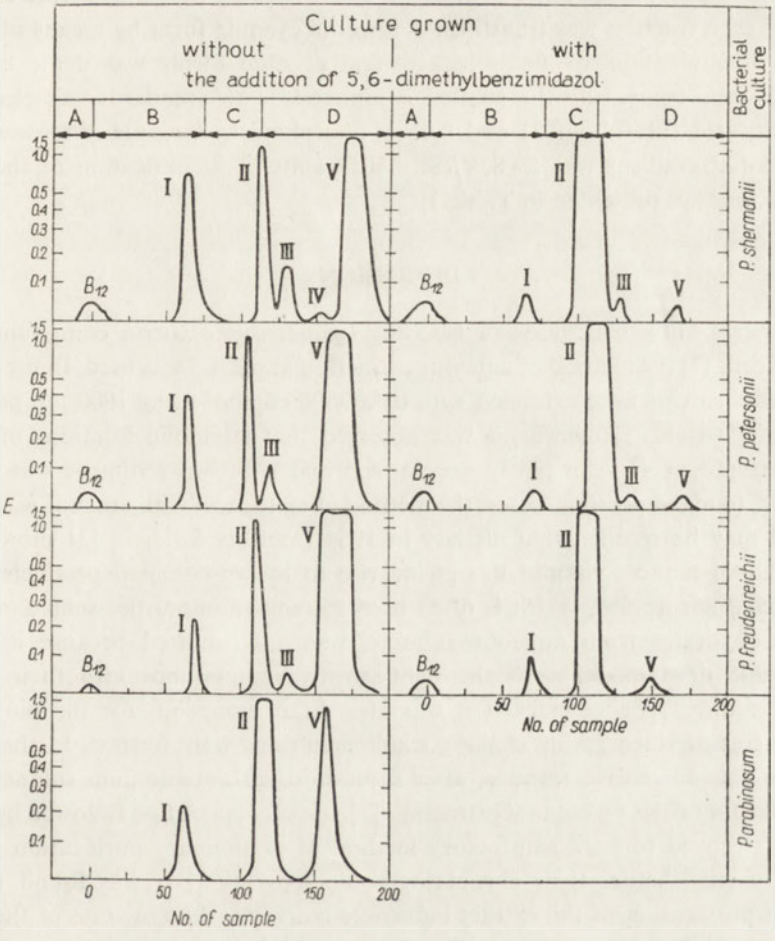

Fig. 1. Chromatographic separation on phosphate-cellulose column of light-sensitive corrinoids isolated from propionic bacteria. (A), Pass through; (B), eluted with 0.005 M-acetate buffer, pH 4.25; (C), pH 5.5; (D), 0.01 M-sodium acetate

the column was washed with water until free of acid. The height of the column was 50 mm. About 150 - 200 ml. of the aqueous solution of corrins was applied to the column. Fractions of 7 ml. were collected by means of an automatic fraction collector. The flow-rate was 7 ml. per 3 - 4 min. After the water had passed through the column, the corrin compounds were eluted successively with: 600 ml. of 0.005

M-acetate buffer, pH 4.25; 200 ml. of 0.005 M-acetate buffer, pH 5.50; and 300 - 500 ml. of 0.01 M-sodium acetate solution. The buffers were prepared from 0.005 M-sodium acetate adjusted with acetic acid to appropriate pH. The concentration of corrins in the fractions was measured with a Spectronic 20 colorimeter, at 460 mμ for yellow-coloured compounds and at 520 mμ for red compounds. To identify the separated fractions (Fig. 1), each one was desalted by phenol extraction and then concentrated on the water bath at 45° under reduced pressure. Before desalting, a part of each fraction was transformed into the cyanide form by means of sodium cyanide. Identification of lightsensitive and cyanide forms was made by paper electrophoresis comparing the studied compounds with standards. As electrolytes 2 M-acetic acid (pH about 3) and 0.05 M-phosphate buffer, pH 6.5, were used. The potential gradient was 5 - 8 V/cm. The results of identification of the individual fractions are presented in Table 1.

DISCUSSION

Of several known methods of isolating light-sensitive corrin compounds from bacterial cells [7] the method of aqueous extraction at pH 4.5 was used. In preliminary experiments corrins were extracted with cold 96% ethanol using 1000 ml. per 300 g. of bacterial weight. However, it was observed that ethanolic solutions of corrins kept overnight at 4° were partly decolorized and a brown sediment was formed. Therefore, to avoid possible losses, the method of extraction with ethanol was abandoned. It may be recalled that already in 1954 Jaselskis & Diehl [3] showed that warm ethanol reduces vitamin B_{12} giving rise to brown-coloured products.

Charcoal adsorption, which is often used to remove impurities such as peptides and carbohydrates from aqueous extracts, was also omitted because it caused considerable decomposition of the light-sensitive compounds into their cyanide forms. The direct phenol extraction was also disadvantageous for the purification of crude corrins since during shaking stable emulsions were formed. In the present work the aqueous corrin extracts, after saturation with ammonium sulphate, were freed from impurities by butanol extraction [7]. Butanol extraction followed by phenol extraction proved to be a satisfactory method of preliminary purification of solutions of corrins before their chromatographic separation. It was found that the degree of purification of the extract influences markedly the flow-rate of the individual fractions from the column. The best separation of S-corrins was achieved on a column 50—55 mm. high; when higher columns were used the tailing of bands was observed in the lower part of the columns.

The elution with 0.005 M-acetate buffer, pH 4.25, resulted in rapid separation of hydroxyl derivatives (formed by decomposition of light-sensitive forms) and acidic light-sensitive corrinoids e.g. Sy2, SB_{12p}(COOH), from SB_{12} or SB_{12p} which are strongly adsorbed by P-cellulose at this pH. For instance, for separation of SB_{12} from the hydroxyl derivative of vitamin B_{12} even a column 10 - 15 mm. high was sufficient. SB_{12} was very well eluted from the column by 0.005 M-acetate buffer

at pH 5.0-5.5, but for the elution of SB_{12p} pH above 6 was necessary. The pH values, volume and concentration of the buffer solutions used for the elution were chosen so as to obtain homogeneous SB_{12} and SB_{12p} fractions, which are the most abundant ones in the bacteria studied. By successive elution with buffers at pH 4.25, 5.50 and 6.5 usually 4 - 5 fractions of light-sensitive corrinoids were obtained. Of these, fraction *I* was the least homogeneous, containing relatively large amounts of monocarboxylic derivatives of SB_{12}, besides Sy2. Fraction *II* contained chiefly SB_{12}, and also $S\psi B_{12}$ and SB_{12}(COOH) (in *P. arabinosum* $S\psi B_{12}$ was the chief compound). SB_{12} was easily separated from $S\psi B_{12}$ by rechromatography of the mixture on P-cellulose, using 0.005 M-acetate buffer, pH 4.0, as eluent. The rechromatographed fraction SB_{12}, after desalting and concentration, crystallized on addition of acetone. The crystalline product contained traces of SB_{12}(COOH). Occasionally, in the presence of a large excess of SB_{12} over the other corrinoids, fraction SFA (*III*) was eluted together with SB_{12} and $S\psi B_{12}$. This compound was easily separated by rechromatography at pH 4.0. Fraction SB_{12p} (*V*) was the most homogeneous one.

The presented method was used for separating the light-sensitive corrinoids from four strains of propionic bacteria grown with or without the addition of 5,6-dimethylbenzimidazole as precursor of SB_{12}. The method can be used for obtaining pure preparations of SB_{12} and SB_{12p}, and for quantitative comparisons of corrinoid composition in different strains of bacteria, e.g. during growth. It was found, for instance, that young cultures of the studied strains contain fairly large amounts of acidic S-corrinoids [SB_{12}(COOH), SB_{12p}(COOH), Sy2] which disappear from older cultures, especially SB_{12}(COOH). The method might also prove useful for taxonomic purposes.

SUMMARY

A method was developed for separating light-sensitive corrinoids on P-cellulose, and was applied to separation of the corrin compounds from four strains of propionic bacteria. The compounds were separated into 4 - 5 fractions, of which fractions SB_{12} and SB_{12p} proved to be the purest ones.

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CHROMATOGRAFICZNY ROZDZIAŁ NA FOSFORANO-CELULOZIE ŚWIATŁOCZUŁYCH FORM KORYNOIDÓW PRODUKOWANYCH PRZEZ BAKTERIE KWASU PROPIONOWEGO

Streszczenie

Opracowano metodę rozdziału światłoczułych korynoidów na P-celulozie. Zastosowano ją do rozdziału związków korynowych czterech szczepów bakterii propionowych. Związki te rozdzielono na 4 - 5 frakcji, z których najbardziej czyste okazały się frakcje SB_{12} i SB_{12p}.

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PREPARATION AND CHEMICAL AND ENZYMIC PROPERTIES OF PHOSPHATE ESTERS OF 1-(β-D-GLUCOPYRANOSYL)URACIL AND -THYMINE

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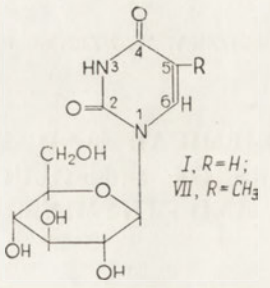
Starting from the first reported chemical synthesis of pyrimidine glycosides by Hilbert *et al.* [13], considerable effort and attention have been devoted to this problem, the most recent useful modification being the application of the mercury method for purine glycosides [5] to pyrimidine analogues [10]. While these methods have been widely applied to the synthesis of nucleosides containing sugars other than ribose and deoxyribose [9], very little attention has been devoted to a study of the phosphorylation products of such nucleosides [3, 15], probably because of the fact that no sugars other than ribose or deoxyribose have hitherto been detected in natural nucleic acids. It nonetheless appeared of interest to us to examine some of the properties of phosphorylated pyrimidine glucosides, including their possible susceptibility to some enzymes involved in nucleotide and nucleic acid metabolism. It is perhaps worth drawing attention to the fact that at least one instance has been reported of the isolation of a glucose nucleotide from natural sources [22a]; further investigation has shown that this nucleotide, which was extracted from cotton plant, and contains glucose, adenine and phosphorus in the ratio 1 : 1 : 1, exhibits the properties characteristic of adenylic acid [22b].

Furthermore the availability of the phosphate ester isomers of pyrimidine glucosides offers the possibility of studying the properties of the 6- and 7-membered cyclic phosphate rings and, in particular, their susceptibility to enzymic hydrolysis.

In addition the availability of the 6′-monophosphate of glucosylurácil opens the way to the preparation of a glucose analogue of UDPG; while such a compound would hardly be expected to exhibit the biological activity of UDPG, it might nonetheless be of interest as a potential antimetabolite.*

* Since the submission of this manuscript, we have been able to show that the glucose analogue of UDPG is inactive in the system leading to the transformation of galactose-1-phosphate to glucose-1-phosphate.

The starting products for synthetic work were 1-(β-D-glucopyranosyl)uracil (*I*), obtained by the method of Hilbert & Johnson [13], and 1-(β-D-glucopyranosyl)-thymine (*VII*), prepared according to the mercury method of Fox *et al.* [10]. The introduction of protective groups on the sugar moiety of such nucleosides for the



purpose of selective phosphorylation is considerably more complex than for the corresponding ribose derivatives because of the absence of any *cis*-glycol system and the presence of three secondary, and one primary, hydroxyl groups.

Monophosphates of glucosyluracil

The known procedure for the introduction of a benzylidene group [3] was applied to *I* to obtain, in good yield, a crystalline derivative which was ascribed the formula 1-(4′,6′-*O*-benzylidene-β-D-glucopyranosyl)uracil (*II*). Phosphorylation of this derivative resulted in the formation of the 2′- and 3′-monophosphates of *I*; the two isomers were separated from each other and from a small quantity of the 2′,3′-diphosphate also formed during the reaction, and identified by means of their reactions with periodate [2].

In order to obtain the 6′-monophosphate of *I*, recourse was had to the well-known method for the etherification of the primary hydroxyl at the 6′-position of *I* with triphenylmethyl chloride, to obtain a crystalline product in almost quantitative yield, 1′-(6′-triphenylmethyl-β-D-glucopyranosyl)uracil. This compound was acetylated and the trityl group then removed [3] to give a crystalline triacetyl derivative of *I*. The corresponding triacetyl derivative of *VII* was obtained by an identical procedure.

Contrary to expectations, phosphorylation of the triacetyl derivative of *I* did not result in the formation solely of the 6′-monophosphate of *I*, but gave two isomers, viz. the 4′- and 6′-monophosphates of *I* (see below for methods of phosphorylation and identification of isomers). Since the same result was obtained even under the very mild phosphorylation conditions accompanying the use of 2-cyanoethylphosphate in anhydrous medium [35], it follows that the removal of the trityl group in 80% acetic acid at elevated temperature must be accompanied by the known migration of acyl groups, predominantly in the direction 4′ → 6′ *via* formation of a transitory

cyclic derivative [29, 23]. Judging from the products of phosphorylation, i.e. the relative amounts of the 4′- and 6′-phosphates of *I* formed (see below and cf. Fig. 1), the triacetyl derivative of *I* must be 1-[2′,3′,4′(6′)-triacetyl-β-D-glucopyranosyl]-uracil (*IV*), the mixture containing about 60% of the 2′,3′,4′-triacetyl derivative and about 40% of the 2′,3′,6′-isomer. It is of interest that, under the experimental conditions employed here, there was observed a migration of acetyl groups only in the direction of the primary hydroxyl; no migration was observed in the direction 2′ → 4′ nor, as would be expected from this, in the direction 3′ → 4′ (*trans*-configuration), since the conditions of more intensive phosphorylation subsequently employed should then have led to the formation of the corresponding monophosphates of *I*. By contrast, no migration of acetyl groups in the direction 3′ → 5′ has been observed in deoxyribonucleosides (see method of preparation of thymidine-5′-phosphate [19]).

The polyphosphate method [12, 18] was applied only in initial small scale experiments. The pyro- and higher phosphates formed during this reaction were hydrolysed in the ordinary manner by boiling in acid, which is also accompanied by removal of the benzylidene blocking group. For the triacetyl derivatives, the acetyl blocking groups were removed by additional boiling for 30 min. at pH 12. The resulting mixture of monophosphate isomers and diphosphates was fractionated on a column of Dowex 2 × 8 (formate form) and the various fractions estimated and identified by means of paper chromatography, ratio of P [8] to molar extinction, and the periodate reaction (see Experimental). However, the foregoing method of phosphorylation did not give unequivocal results. In particular the expected quantities of isomeric monophosphates were not obtained, e.g. in the case of *II* more

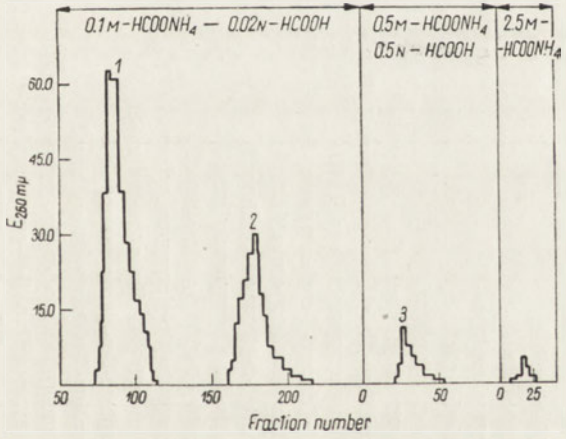


Fig. 1. Fractionation of products of phosphorylation of 1-[2′,3′,4′(6′)-triacetyl-β-D-glucopyranosyl]uracil on Dowex 2 × 8 (formate form): 705 mg. of phosphorylation products (after freeing of Ba^{2+}) deposited on a 40×1.5 cm. column and eluted as shown on diagram, fractions of 12 ml. being collected at 10-min. intervals, suitably diluted and spectrophotometered at 260 mμ. (*1*), Glucosyluracil-6′-phosphate, (*2*), -4′-phosphate, (*3*), -4′,6′-diphosphate

Table 1

Ascending paper chromatography on Whatman paper no. 1

Solvent systems: (*A*), ethanol - 1 M-ammonium acetate (5:2, v/v); (*B*), propan-2-ol - ammonia (d = 0.88) - water (7:1:2, by vol.); (*C*), ethanol - 0.5 M-ammonium acetate (5:2, v/v).

Compound	R_F in solvent		
	A	*B*	*C*
Uridine	0.73	0.49	—
Uridine-2′(3′)-phosphate	0.25	0.17	—
Uridine-5′-phosphate	0.26	0.17	0.40
Uridine-5′-pyrophosphate	0.12	—	—
Uridine-2′:3′-phosphate	—	0.29	—
Uridine diphosphate glucose	—	—	0.27
1-(β-D-Glucopyranosyl)uracil (*I*)	0.60	0.38	—
1-(4′:6′-Benzylidene-β-D-glucopyranosyl)uracil (*II*)	0.85	—	—
1-(6′-Trityl-β-D-glucopyranosyl)uracil (*III*)	0.80	—	—
1-[2′,3′,4′(6′)-Triacetyl-β-D-glucopyranosyl]uracil (*IV*)	0.91	—	—
1-(2′-Phosphate-β-D-glucopyranosyl)uracil	0.25	0.07	—
1-(3′-Phosphate-β-D-glucopyranosyl)uracil	0.25	0.08	—
1-(4′-Phosphate-β-D-glucopyranosyl)uracil	0.25	0.07	—
1-(6′-Phosphate-β-D-glucopyranosyl)uracil	0.25	0.05	0.32
1-(6′-Pyrophosphate-β-D-glucopyranosyl)uracil (*V*)	0.12	—	—
1-(2′,3′-Diphosphate-β-D-glucopyranosyl)uracil	0.10	—	—
1-(2′:4′-Phosphate-β-D-glucopyranosyl)uracil	—	0.25	—
1-(3′:6′-Phosphate-β-D-glucopyranosyl)uracil	—	0.25	—
1-(6′-Phosphomorpholidate-β-D-glucopyranosyl)uracil	—	—	0.50
1-(β-D-Glucopyranosyl)uracil-6′-pyrophosphate-glucose (*VI*)	—	0.05	0.21
1-(β-D-Glucopyranosyl)thymine (*VII*)	0.60	—	—
1-[2′,3′,4′(6′)-Triacetyl-β-D-glucopyranosyl]thymine (*VIII*)	0.90	—	—
1-[6′(4′)-Phosphate-β-D-glucopyranosyl]thymine (*IX*)	0.27	0.06 0.07	—
Glucose	—	0.45	—
Glucose-1-phosphate	—	0.05	—
Glucose-6-phosphate	—	0.05	—
Glucose-4:6-phosphate	—	0.26	—

than the expected two isomers were found (see Experimental), due undoubtedly to the rather drastic phosphorylation conditions or the subsequent operations involved in isolation of the reaction products. It was therefore decided to conduct further phosphorylations, on a larger scale, with the use of 2-cyanoethylphosphate as phosphorylating agent.

The phosphorylating agent, and the method of its application, were as described by Tener [35]; phosphorylation times were, however, shortened in order to reduce to a minimum the quantity of diphosphate formed. The products were separated

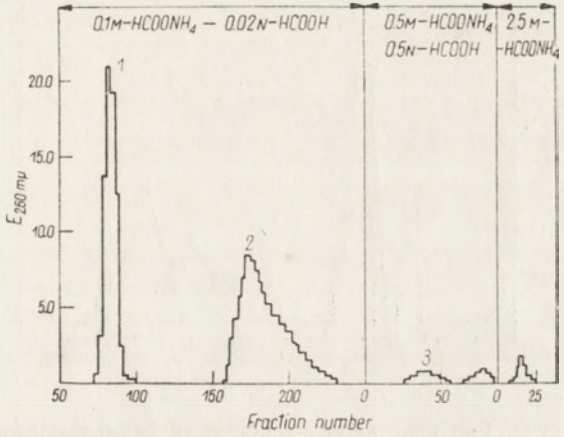


Fig. 2. Column fractionation on Dowex 2 × 8 (formate form) of phosphorylation products of 1-(4′:6′-benzylidene-β-D-glucopyranosyl)uracil: 251 mg., freed from Ba^{2+}, deposited on a 40 × 1.5 cm. column and eluted as shown in figure. Fractions of 12 ml. collected at 10 min. intervals, suitably diluted and extinction determined at 260 mμ

(*1*), Glucosyluracil-2′-phosphate, (*2*), -3′-phosphate, (*3*), -2′,3′-diphosphate.

on a Dowex 2 × 8 formate column. Phosphorylation of *II* led, as expected, to the formation of two monophosphates, and a small quantity of the diphosphate, of *I* (see Fig. 2). The monophosphate isomers were identified by quantitative periodate oxidation, by determinations of P to molar extinction in the UV, and by paper chromatography (see Table 1). From these determinations it followed that the first peak in Fig. 2 corresponds to the 2′-monophosphate of *I* and the second peak to the 3′-phosphate.

On the other hand, and contrary to expectations, phosphorylation of *IV* resulted in the formation of two monophosphates, and small quantities of the diphosphate, of *I* (see Fig. 1). The two monophosphates were identified as described above, from which it resulted that the first peak in Fig. 1 corresponded to the 6′-phosphate of *I* since only this isomer consumed two moles of periodate. The compound corresponding to the second peak in Fig. 1 was identified by fractionation together with the previously identified 2′- and 3′-monophosphates of *I* on a Dowex column. Since it was not eluted together with either of the known monophosphates

(Fig. 3), while its absorption spectrum was characteristic for *I*, and the ratio of P to molar extinction was 1, it can be only the 4′-monophosphate of *I*. With a view to determining the position of elution of the 6′-phosphate of *I* with respect to the other isomers, it was eluted from a column together with a mixture of the 2′- and 3′-isomers of *I* under the conditions indicated in Fig. 3. It is clear from the Figure that the 6′-phosphate is eluted from a Dowex 2 × 8 formate column ahead of the other isomeric phosphates of *I*. The situation is therefore analogous to that pre-

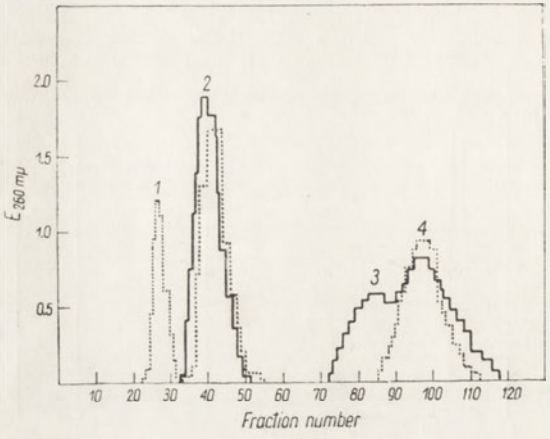


Fig. 3. Fractionation of the four monophosphate isomers of 1-(β-D-glucopyranosyl)uracil (*I*) on Dowex 2×8 (formate form); column size 10×1 cm.; (—), mixture of 2′-, 3′- and 4′-phosphates of *I*; (...), mixture of 2′-, 3′- and 6′-phosphates of *I*. The 4′- and 6′-phosphates were placed on column in quantities of 1 mg., and the 2′- and 3′-phosphates in quantities of 2 mg. Elution by means of 0.05 M-ammonium formate + 0.01 N-HCOOH. Fractions of 5 ml. collected at 10 min. intervals, suitably diluted and spectrophotometered at 260 mμ. (*1*), Glucosyluracil-6′-phosphate, (*2*), -2′-phosphate, (*3*), -4′-phosphate, (*4*), -3′-phosphate

vailing for the corresponding ribose phosphates, where the first peak is due to the 5′-phosphate, i.e. the isomer in which the primary hydroxyl is esterified. The elution sequence for the isomeric monophosphates of *I* from a Dowex 2 × 8 column under standard conditions (Fig. 3) is therefore as follows: 6′-, 2′-, 4′-, 3′-.

6′(4′)-Monophosphate of glucosylthymine

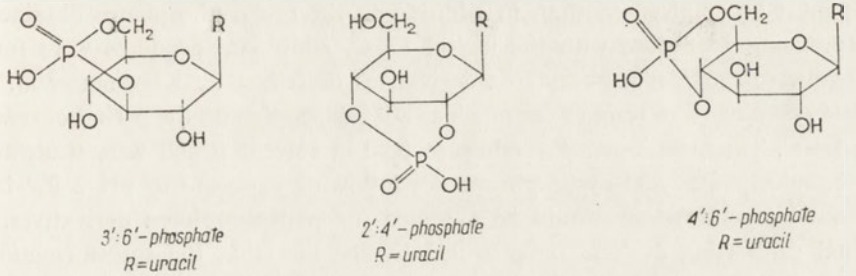
This was prepared as for the corresponding monophosphate of *I* by tritylation, acetylation and then detritylation of glucosylthymine, followed by phosphorylation according to the polyphosphate method. The final product was not homogeneous, but exhibited a tendency to resolve into two spots in solvent *B*. By analogy with the corresponding situation for glucosyluracil (see above) this is most likely due to the presence of a mixture of the 4′- and 6′-monophosphates of *VII*. As will be shown below, this is indirectly supported by the action of 5′-nucleotidase on this substance.

6′-Pyrophosphate of glucosyluracil

This was prepared by phosphorylation of the mono-(tri-*n*-octylamine)-6′-phosphate of *I* by means of dibenzylphosphochloridate [16, 18].

Cyclic phosphates

Three of the isomeric monophosphates of *I*, the 2′-, 3′- and 6′-, were employed for the preparation of the cyclic phosphates. An examination of molecular models, as well as reported findings on the cyclic phosphates of glucose (2, 17, 30] and of the 5′-phosphates of natural [27] and synthetic [36] nucleotides suggested that one might expect the formation of 6- and 7-membered phosphate rings.



Cyclic phosphate esters of 1-(β-D-glucopyranosyl)uracil

Although cyclization was carried out principally by means of dicyclohexylcarbodiimide (DCC) in aqueous pyridine [17], several trials were conducted with DCC in anhydrous medium and with trifluoroacetic anhydride. The results with these two latter procedures, while less effective, were nonetheless of assistance in deducing the nature of the products of cyclization. For the sake of simplicity, they will be discussed at the end of this section.

The products of cyclization were separated by means of paper chromatography. For each of the three monophosphate isomers, two main reaction products were formed, one with an R_F value about 0.9 (see next paragraph) and the other with an R_F value very close to that for uridine-2′:3′-phosphate. The latter was identified as a cyclic phosphate of *I*; it had a ratio of P to molar extinction of 1 and was completely resistant to acid monophosphatase. Taking into account steric factors, the product of cyclization of the 2′-phosphate of *I* would be the 2′:4′-phosphate of *I*, while the product of cyclization of the 3′-phosphate should be the 3′:6′-phosphate of *I*. Since it did not prove feasible to differentiate between the rates of formation or the properties of 6-membered (2′ : 4′-) and 7-membered (3′ : 6′-) cyclic phosphates, both of them exhibiting the same R_F values chromatographically and similar resistance to chemical hydrolysis, it was not possible to establish whether the product(s) of cyclization of the 6′-phosphate of *I* was the 4′ : 6′ or 3′ : 6′ or

a mixture of both. As will be shown below, the cyclic phosphates are hydrolysed to monophosphates by some tissue enzymes. A combination of enzymic hydrolysis and column chromatography of the resulting monophosphates, as already described above, might assist in the identification of the original cyclic phosphates. This will, of course, be true only in those instances where a given cyclic phosphate is not hydrolysed to the parent monophosphate from which it was prepared. It is planned to explore this possibility.

All the cyclic phosphates obtained underwent complete decyclization in 0.5 N-HCl in 5 - 6 hr. at 100°, with the formation of monophosphates of *I* which were readily dephosphorylated by monophosphatase. These findings confirm those of other workers [2, 17, 30] with regard to the increased stability of 6- and 7-membered rings, as compared to 5-membered ones but do not provide any information as to the relative stabilities of 6- and 7-membered rings.

It must be emphasized that, in addition to the cyclic phosphates obtained by the foregoing procedure with the use of DCC, additional products were formed analogous to the *N*-phosphorylurea derivatives described by Khorana *et al.* [17], in yields ranging from traces to as much as 20% depending on the period of reaction with DCC. These exhibited R_F values of 0.90 in solvent *B* and were transformed to monophosphates and cyclic phosphates following exposure to pH 2 for 18 hr. at room temperature, as would be expected for phosphorylurea derivatives. Additional supporting evidence is provided by the fact that the transformation of these derivatives at pH 2 to cyclic and monophosphates was accompanied by the appearance of a colourless precipitate insoluble in water and soluble in ether; on a paper chromatogram this substance reacted with *p*-dimethylaminobenzaldehyde to give the typical yellow colour characteristic for urea derivatives [7]. This precipitate therefore corresponds to dicyclohexylurea. It must be emphasized that the phosphorylureas of the corresponding ribose derivatives are, under identical conditions, transformed exclusively to the cyclic phosphates, with no evidence of formation of monophosphates [12, 26]. It should be pointed out that Khorana *et al.* [17] observed the production of such compounds only in reactions leading to the formation of 5-membered cyclic phosphates*, it being concluded that this is related to the greater lability of the latter as compared to 6- and 7-membered rings. In our experiments, on the other hand, the phosphorylurea derivatives are apparently formed notwithstanding that the formation of 5-membered rings from monophosphates of *I* is excluded.

It is consequently doubtful whether one may draw any general conclusions as to the stability of cyclic phosphate rings on the basis of their size alone. The stability is undoubtedly also dependant on the nature of the ring, e.g. whether we are dealing with a furanose or pyranose ring in the case of carbohydrates, or

* Attention is directed to the fact that cyclization of the ammonium salts of cytidine-2′(3′)-phosphate and adenosine-2′(3′)-phosphate by the action of DCC in anhydrous methanol proceeds quantitatively without formation of any side products [26].

with an aliphatic compound, as for 1,4-butane-diol or 1,3-propane-diol [17]. For the particular class of compounds dealt with in this work, the stability of the cyclic phosphates is also dependant on the nature of the aglycone. An excellent illustration of this is guanosine-2′:3′-phosphate, which is considerably more labile in aqueous medium than the other ribonucleoside cyclic phosphates [25, 34, 28].

In addition to the reaction products described above, it was observed that the cyclization reactions are accompanied by the appearance of several other products, one of them transitory, in varying yields. The quantities were, however, too small to permit of their identification (see Experimental for details).

In view of the overall complexity of the cyclization products obtained, it is worth drawing attention to several other pertinent observations.

It has been reported by Khorana *et al.* [17] that, under the conditions employed above for cyclization, the conversion of glucose-6-phosphate to glucose-4:6-phosphate is essentially quantitative. This finding is somewhat difficult of interpretation in view of the variety of products obtained with monophosphates of glucosyluracil. The cyclization of glucose-6-phosphate was consequently carried out as described by Khorana *et al.* [17] and it was confirmed chromatographically that only one product did indeed result in practically quantitative yield of the cyclic phosphate.

Attention was then directed to the use of DCC in dimethylformamide, which has been shown to result in a quantitative conversion of uridine-2′(3′)-phosphate to uridine-2′:3′-phosphate and uridylurea derivatives. On addition of water to the medium, the latter are also converted to the cyclic phosphates, so that the only end product is uridine-2′:3′-phosphate [26]. However, when this procedure was applied to a mixture of the 4′- and 6′-monophosphates of *I*, the reaction products were identical to those obtained by the use of aqueous pyridine as the reaction medium, but in considerably lower yield. Because of this latter fact, the use of dimethylformamide as reaction medium was discontinued.

Several attempts were also made to obtain the cyclic phosphates with the use of trifluoroacetic anhydride as described by Brown *et al.* [4]. The products obtained in this case were again similar to those resulting from the action of DCC in aqueous pyridine with this exception, that the product with R_F value about 0.90, which we consider to be a phosphorylurea derivative, was completely absent. This provides reasonably good evidence that this product is indeed a phosphorylurea derivative. However, the overall yields here were also considerably lower than with the DCC--pyridine method and the use of trifluoroacetic anhydride was consequently also abandoned.

Glucose analogue of UDPG: 1-(β-D-glucopyranosyl)uracil-6′-pyrophosphate-glucose

The preparation of this analogue of UDPG, in which the uridine moiety is replaced by glucosyluracil, was carried out as described for UDPG by Roseman *et al.* [24], involving the condensation of the 6′-phosphomorpholidate of *I* with the tri-*n*-octylamine salt of glucose-1-phosphate in anhydrous pyridine. The reaction

products were fractionated on a Dowex 1 × 8 column, elution being carried out with LiCl and HCl. The isolated coenzyme analogue exhibited an R_F in solvent *A* similar to that for UDPG, an UV spectrum similar to that for *I*, and a behaviour towards acid hydrolysis similar to that for UDPG. Heating for 10 min. at 100° in 0.01 N-HCl liberated glucose and the 6′-pyrophosphate of *I*; under more drastic hydrolysis conditions (30 min. at 100° in 1 N-HCl), the hydrolysis products were glucose and the 6′-monophosphate of *I*.

Enzymic dephosphorylation of monophosphates

All of the monophosphates of *I* and *VII* were quantitatively dephosphorylated to *I* and *VII* by intestinal and prostate monophosphatases, as might be expected. Somewhat unexpected, however, was the finding that the 6′-phosphates of both *I* and *VII* underwent partial dephosphorylation on treatment with snake venom, the 6′(4′)-phosphate of *VII* being particularly susceptible. Incubation at 37° for 24 hr. resulted in the formation of about 60 - 70% of *VII*, the remaining 30 - 40% presumably corresponding to the amount of the 4′-isomer present in the monophosphate preparation. This is supported by the finding that the 4′-phosphate of *I*, which was separated as described above from the mixture of 6′- and 4′-phosphates, was resistant to snake venom. The foregoing results are all the more striking in that, in agreement with observations in the literature to the effect that glucose-6--phosphate is resistant to 5′-nucleotidase, it was found that glucose-6-phosphate was unaffected by snake venom. Additional control experiments showed that ribonucleoside 5′-phosphates were rapidly and quantitatively hydrolysed to the corresponding nucleosides by the snake venom.

While it would be highly desirable to confirm the above findings with purified 5′-nucleotidase, the apparent ability of this enzyme in snake venom to attack the 6′-monophosphates of *I* and *VII* raises some important problems with regard to the specificity of an enzyme which has hitherto been regarded as highly specific.

Enzymic hydrolysis of cyclic phosphates

An examination was also made of the susceptibility to enzymic hydrolysis of the cyclic phosphates formed from the 2′-, 3′- and 6′-monophosphates of *I*. All of these cyclic phosphates proved to be resistant to the action of pancreatic ribonuclease, a result which is not unexpected. If we regard the catalytic activity of ribonuclease as a specific example of acid-catalysed hydrolysis [37, 33], it is to be expected that the resistance of the foregoing cyclic phosphates, containing 6- or 7-membered rings, is related to their increased resistance to acid hydrolysis as compared to the 5-membered ribose cyclic phosphates. Furthermore the location of the number 3 nitrogen of the uracil ring with respect to the carbohydrate moiety which, as has been previously shown [31, 32] is of considerable significance in relation to ribonuclease action, is different in the case of 1-(β-D-glucopyranosyl)uracil as compared to 1-(β-D-ribofuranosyl)uracil.

All the cyclic phosphates of *I* were also found to be resistant to the phosphodiesterase of snake venom, notwithstanding that snake venom slowly hydrolyses uridine-3′:5′-phosphate [27], which contains a 6-membered cyclic phosphate ring.

On the other hand it was found that the various cyclic phosphates of *I* are transformed to monophosphates by extracts of rabbit brain, such as those used by Drummond & Perrott-Yee [6] to decyclize the 3′:5′ cyclic phosphates of adenosine, guanosine and uridine, as well as by calf liver extracts. The brain extracts exhibited much higher enzymic activity, against the cyclic phosphates of *I*, as compared to the liver extracts, calculated on the basis of the protein content of the extracts. The products of action of the extracts included, in addition to the monophosphates of *I*, the nucleoside *I* itself when incubation was sufficiently prolonged, due to the action of monophosphatases present in the extracts. Both the brain and liver extracts proved to be inactive in the presence of EDTA (sodium versenate). Since the addition of EDTA does not inhibit the decyclization of uridine-2′:3′-phosphate by the foregoing extracts [6], it would appear that a different enzyme is involved in the decyclization of the cyclic phosphates of *I*. It remains to establish whether this enzyme is specific for such cyclic phosphate rings and what is the extent of its specificity. It was found that the same extracts would also effectively decyclize glucose-4:6-phosphate to give glucose-6(4?)-phosphate and glucose. It is consequently conceivable that we are dealing with some non-specific enzyme, and further experiments are necessary to establish this point. The existence of such an enzyme may not be without significance in relation to the role of glucose-6-phosphate in intermediary metabolism. It will be of interest to determine which of the isomeric phosphates are the products of enzymic hydrolysis of the various cyclic phosphates (see page 328); this would assist in deciding whether more than one enzyme is involved.

EXPERIMENTAL

1-(4′,6′-Benzylidene-β-D-glucopyranosyl)uracil (*II*). To 1.5 g. *1-(β-D-glucopyranosyl)uracil* (*I*), prepared according to the method of Hilbert & Johnson [13] and dried under reduced pressure at 90°, was added 25 ml. benzaldehyde and 3 g. anhydrous $ZnCl_2$. The reaction mixture was shaken at room temperature for 48 hr., then added to 600 ml. ether and left standing for several hours. The resulting precipitate was filtered off, washed with ether and dissolved in 50 ml. methylcellosolve. To this solution was added 8 ml. 5 N-NaOH and, after a 20 min. interval, CO_2 was bubbled through the solution until it was neutral. The resulting precipitate was filtered off and washed with hot methyl cellosolve. The combined filtrates were evaporated under reduced pressure to about 5 ml. and 25 ml. water added to produce a precipitate which was washed with water and crystallized from 90% ethanol. The final product, *II*, 1.49 g. (75.3% theor.) exhibited only one spot in solvent *A* and with *n*-butanol - NH_4OH (d = 0.88), (45 : 5, v/v); m.p. 275 - 276° (decomp.). Calculated for $C_{17}H_{18}O_7N_2$: N, 7.74%; found N, 7.47%.

2′ and 3′ monophosphates of 1-(β-D-glucopyranosyl)uracil. (a) Initial phosphorylation of *II* was carried out with a mixture of P_2O_5 in 85% H_3PO_4 [12, 18] at a temperature of 60° for 45 min. Water was then added and the resulting solution heated on a boiling water bath for 60 min., neutralized by means of $Ba(OH)_2$ and, following filtration of the precipitated barium phosphate, the barium salts of the mononucleotides were precipitated by addition of two volumes of ethanol.

The mixture of mononucleotides was chromatographed on a 11 × 1 cm. column of Dowex 2 × 8 (formate form) under conditions similar to those indicated in Fig. 3. Elution gave three main peaks, for which the ratio of P to molar extinction was 1, followed by a fourth peak which consisted principally of diphosphates (as shown by paper chromatography) and other unidentified products. Peak *1* contained 16% of the total material and was identified as the 6′-phosphate of *I*. Peak *2* contained 17% of the total material and corresponded to the 2′-phosphate of *I*. Peak *3* was less sharp, consisting of a poorly resolved mixture of the 4′- and 3′-phosphates of *I*, as inferred from subsequent experiments, described below, where elutions were carried out precisely as described in Fig. 3.

(b) To 540 mg. (1.5 m-mole) of *II* was added 9 ml. (9 m-mole) of a solution of cyanoethylphosphate in pyridine and water, and the solvents removed under reduced pressure. The residue was taken up in 30 ml. anhydrous pyridine, which was then removed under reduced pressure at 30°, and this operation was repeated once. The resulting anhydrous residue was dissolved in 40 ml. anhydrous pyridine, 5 g. DCC added, and the reaction mixture left for 30 hr. at 29.° About 5 ml. water was then added and, after 1 hr., the precipitated cyclourea was filtered off and the filtrate evaporated to dryness. The residue was then hydrolysed in 30 ml. 10% acetic acid for 90 min. at 100° and, following removal of the acid by evaporation at reduced pressure, in 30 ml. 9 N-ammonia for 90 min. at 60°.

The ammonia was distilled off and the residue taken up in 10 ml. water, which was then freed of acrylonitrile and some cyclohexylurea by filtration. The filtrate was freed of cations by shaking with Amberlite IR-120(H^+) and then brought to pH 7.5 with $Ba(OH)_2$. The precipitate which formed was filtered off and washed with water, the combined filtrates concentrated under vacuum to 3 ml., and 6 ml. ethanol added to precipitate the barium salts of the mononucleotides, which were separated by centrifugation, washed with ethanol and ether and dried. Chromatography in solvent *A* showed the product to be free of nucleoside and contaminated with only a small quantity of diphosphates; yield 69%, determined spectrophotometrically.

The product was dissolved in water, Ba^{2+} ions removed by shaking with Amberlite IR-120(H^+), and the solution brought to pH 9 with ammonia and deposited on a column of Dowex 2 × 8 (formate form). Results are shown in Fig. 2. For fractionation of 751 mg. (0.71 m-mole) nucleotide, a column 40 × 1.5 cm. was employed. Monophosphates were eluted with 0.1 M-$HCOONH_4$ and 0.02 N-HCOOH and diphosphates with 0.5 M-$HCOONH_4$ and 0.5 N-HCOOH, fractions of 12 ml. being collected at 10 min. intervals and spectrophotometered at 260 mμ. Of the

total material, 92% was eluted in two fractions with the dilute buffer, the first peak containing 40%, the second 52%. The more concentrated buffer was then used to remove an additional 6% in one peak, and the final 2% was eluted with 2.5 M-$HCOONH_4$. The individual fractions were brought to pH 9 with ammonia and readsorbed on small columns consisting of 1 ml. Dowex 2 × 8 (Cl^-). Following percolation of some water through the columns, the substances were eluted with small quantities of 0.1 N-HCl and the resulting solutions evaporated under reduced pressure.

Fractions 1 and 2 exhibited a ratio of P [8] to molar extinction of 1, and were transformed quantitatively to *I* by means of prostate phosphatase. Both fractions were subjected to periodate oxidation in 1 M-acetate buffer, pH 3.2, for 24 hr. in the dark [2]. Fraction 1 took up 1 mole periodate, while fraction 2 was completely resistant. On the basis of the foregoing, fraction 1 is identified as the 2′-phosphate of *I* and fraction 2 as the 3′-phosphate of *I*.

1-(6′-Trityl-β-D-glucopyranosy)uracil (III). To 1.7 g. (6.22 m-mole) of 1-(β-D-glucopyranosyl)uracil, dried at 90° under reduced pressure and dissolved by heating to 100° in 125 ml. anhydrous pyridine, was added 1.75 g. (6.3 m-mole) triphenylmethyl chloride. The mixture was heated for 3 hr. at 100° with exclusion of moisture. The entire reaction mixture was then added, with vigorous stirring, to 600 ml. water at 0°. The resulting white precipitate was filtered off and washed several times with ice-cold water, and dried. Yield of *III*, 3.05 g. (95% theor.); following crystallization from anhydrous ethanol, m.p. 253 - 254°. For $C_{29}H_{28}O_7N_2$, N calculated 5.42%, N determined 4.9%.

1-[2′,3′,4′(6′)-Triacetyl-β-D-glucopyranosyl]uracil (IV). To 2 g. of *III*, dried at 90° under reduced pressure and dissolved in 20 ml. anhydrous pyridine, was added 12 ml. of freshly distilled acetic anhydride. The solution was heated for 30 min. at 100° and then left overnight at room temperature, with exclusion of moisture. The solution was then added, with vigorous stirring, to 320 ml. ice water. The resulting white, gummy, precipitate was filtered; during repeated washing with water it was transformed into a finely crystalline mass. Yield, after drying, of 1-(2′,3′,4′-triacetyl-6′-trityl-β-D-glucopyranosyl)uracil, 2.48 g. (100% theor.). The product was dissolved in 50 ml. of 80% acetic acid and boiled under reflux for 30 min. The reaction mixture was cooled and added to 200 ml. ice water, to give a precipitate which was removed by filtration. The filtrate was evaporated under reduced pressure to give an oil which was taken up in ethanol and crystallized by addition of ether. Yield of *IV*, 1.38 g. (90% theor.); m.p. 98 - 102°. For $C_{16}H_{20}N_2O_{10}$, N calculated 7.0%, N measured 7.05%. The compound was chromatographically homogeneous in solvent *A* and, following hydrolysis at pH 9 and 100°, was transformed quantitatively to *I*.

4′- and 6′-monophosphates of I. (a) To 1.2 g. (3 m-mole) of *IV* was added 10 ml. (10 m-mole) of a solution of cyanoethylphosphate in pyridine and water. Following removal of solvents by evaporation, the residue was evaporated three times from dry pyridine to give an oily product which was dissolved in 30 ml. anhydrous pyridine,

to which was added 5 g. DCC. The reaction mixture was left for 2 days at 29°. About 3 ml. water was then added and, after 1 hr., the precipitated cyclohexylurea was filtered off. The resulting yellow solution was evaporated to dryness under vacuum. The residue was then hydrolysed in 30 ml. 9 N-NH_4OH for 90 min. at 60° and, following removal of the ammonia, taken up in several ml. water and the precipitate of acrylonitrile removed by filtration. The filtrate was freed of cations by treatment with 3 g. of Amberlite IR-120(H^+), concentrated under vacuum to several ml., brought to pH 7.5 with hot $Ba(OH)_2$ and the solution again clarified by filtration to remove barium phosphate. The barium salts of the mononucleotides were then precipitated by addition of 2 vol. ethanol and washed with ethanol and ether. Yield, determined spectrophotometrically, 60% theor. Chromatography in solvent *A* showed the absence of nucleoside, and the presence of only slight quantities of diphosphates. In solvent *B* the mononucleotides, isolated as a single spot in solvent *A*, were barely resolved into two spots, presumably the 4′- and 6′-monophosphates.

The foregoing product was fractionated on a column of Dowex 2 × 8 (formate form), see Fig. 1, the procedure being identical with that used for separation of 2′- and 3′-phosphates. In place of the expected single peak corresponding to the 6′-phosphate of *I*, elution with 0.1 M-$HCOONH_4$ and 0.02 N-HCOOH gave two peaks, no. 1 of which embraced 50% of the total material deposited on the column, while no. 2 contained 40%. Further elution with 0.5 M-formate and 0.5 N-formic acid gave peak no. 3 which contained 9% of the eluted material. The various fractions were freed from salt and isolated as described above for the 2′- and 3′-phosphates. For fractions 1 and 2, the ratio of P to molar extinction was 1; for fraction 3 the corresponding ratio was 2.

Further identification of the foregoing monophosphates (fraction 1 and 2) was obtained by mixing each with the 2′- and 3′-phosphates obtained above, followed by column chromatography. Identification was made more positive by using 1 mg. quantities of fractions 1 and 2 and 2 mg. quantities of the 2′- and 3′-phosphates. Each mixture was chromatographed in an identical manner; it was first brought to pH 9 with ammonia and diluted to a concentration of 0.01 m-mole/ml, then applied on a 11 × 1 cm. column of Dowex 2 × 8 (formate form). The column was then washed with water and elution carried out with 0.01 M-$HCOONH_4$ and 0.01 N-HCOOH, 5-ml. fractions being collected at 10-min. intervals. The concentrations of the individual fractions were determined spectrally. Both of the foregoing monophosphates were clearly separated from the 2′- and 3′-isomers (Fig. 3).

Both monophosphate fractions were subjected to periodate oxidation. Only one of them, fraction 1, underwent oxidation with uptake of 2 moles periodate and consequently is the 6′-phosphate of *I*. Fraction 2 is therefore the 4′-phosphate of *I*.

(b) To 200 mg. of *IV* was added 12 ml. of phosphorylation mixture (containing P_2O_5 - 85% H_3PO_4 in the ratio 3 : 4) and the reaction vessel shaken on a wrist-action vibrator for 9 hr. at 60° with exclusion of moisture. Chromatography at

this point exhibited the presence of 50% of unreacted *IV*. Following addition of 40 ml. water, the entire solution was heated for 30 min. at 100°, cooled and brought to pH 7.2 by addition of saturated barium hydroxide. The precipitated barium phosphate was filtered off and washed with water. The combined filtrates were concentrated, brought to pH 12 with 1 N-NaOH and heated for 30 min. at 100°. The pH was then brought to 8.4, some insoluble material filtered off, the filtrate concentrated to about 1 ml. and two volumes ethanol added. The resulting precipitate was washed with ethanol, ether, and then dried.

The product was chromatographed in solvent *B* and, like the product of cyanoethylphosphate phosphorylation, exhibited two barely resolvable spots of monophosphates.

1-(6'-Pyrophosphate-β-D-glucopyranosyl)uracil (V). Following the procedure described by Michelson [18], 44 mg. (0.12 m-mole) of the 6'-phosphate of *I* was phosphorylated to obtain 28 mg. of the Ca salt of the 6'-phosphate of *I* (yield 52% theor.). The product was chromatographically homogeneous in solvent *A* and a solvent consisting of propane-2-ol - 1% $(NH_4)_2SO_4$, (60:40, v/v) [1]. On heating at 100° in 1 N-HCl, the product was quantitatively transformed to the 6'-monophosphate of *I* in 30 min.

1-[2',3',4'(6')-Triacetyl-β-D-glucopyranosyl]thymine (VIII). Crude 1-(β-D-glucopyranosyl)thymine (*VII*) was prepared by the mercury procedure [10] and was freed from small impurities by elution from a cellulose column with water-saturated butanol. To 130 mg. (0.45 m-mole) of *VII*, dried at 90° under reduced pressure, and dissolved in 3 ml. anhydrous pyridine by heating to 100°, was added 125 mg. (0.45 m-mole) triphenylmethyl chloride. The reaction mixture was heated for 3 hr. at 100° with exclusion of moisture, and then poured, with vigorous stirring, into 30 ml. ice water. The resulting white precipitate was separated by filtration, washed with water, and dried to give 240 mg. (100% theor.) of 1-(6'-trityl-β-D-glucopyranosyl)thymine. This was dissolved in 5 ml. dry pyridine, to which was added 5 ml. freshly distilled acetic anhydride, and the mixture left at room temperature overnight. It was then added to 100 ml. ice water to give a precipitate which was washed with water and dried. Yield of 1-(2',3',4'-triacetyl-6'-trityl-β-D-glucopyranosyl)thymine, 290 mg. (100% theor.). The product was dissolved in 10 ml. 80% acetic acid and boiled for 30 min., then cooled and poured into 100 ml. ice water. The resulting precipitate was removed and the filtrate evaporated to give a product which was crystallized from ethanol by addition of ether. Yield 150 mg. (79% theor.) of *VIII*, m.p. 119 - 123°. The product was chromatographically homogeneous in solvent *A* and, following hydrolysis at pH 9 and 100°, was transformed quantitatively to *VII*. For $C_{17}H_{22}N_2O_{10}$, N calculated 6.7%; N obtained 7.31%.

1-[6'(4')-Phosphate-β-D-glucopyranosyl]thymine (IX). 130 mg. of *VIII* was mixed with 2 ml. of the P_2O_5 - H_3PO_4 phosphorylating mixture and vigorously shaken for 3 hr. at 60°, at which time chromatography exhibited formation of *IX* in about 50% yield. The product of phosphorylation was isolated in the same way as for 1-[6'(4')-phosphate-β-D-glucopyranosyl]uracil (30% theor. yield). It was

chromatographically homogeneous in solvent *A*, barely resolvable into two spots in solvent *B*, and was quantitatively transformed to *VII* by prostate or kidney monophosphatase.

Cyclization of 2'-, 3'- and 6'-phosphates of I. For preparation of the cyclic phosphates, each of the isomeric monophosphates was first freed of Ba^{2+} ions by means of Amberlite IR-120(H^+) and lyophilized. The final method for cyclization was that of Khorana *et al.* [17], with DCC in 80% pyridine. The course of the reaction was followed by means of paper chromatography with solvent *B*; and was usually terminated, after shaking the reaction vessel for 72 hr. at room temperature, by addition of water. The precipitated cyclourea was then filtered off, the filtrate extracted twice with ether and the products separated on a small scale by chromatography with solvent *B* on Whatman no. 3MM, from which the individual spots were eluted and concentrated under vacuum.

For all the isomeric monophosphates, there remained about 10% of unreacted substance. The main product of cyclization, in 50 to 70% yield, exhibited an R_F of 0.25; in addition there was a 20% yield of cyclohexylurea derivatives with an R_F of 0.90. Prolongation of the reaction beyond 72 hr., even following addition of water, resulted in an increase in the yield of cyclohexylurea derivatives.

In addition, for short reaction periods, there appeared in yields up to 10% a product with R_F 0.38 which was very labile, and which was transformed to another unidentifiable product with R_F 0.55 during the course of the reaction. The latter product also was formed directly. It contained no phosphorus, was stable to acid, and its absorption spectrum, which was similar to that for glucosyluracil, was unchanged on alkalization, showing that the number 3 nitrogen was blocked.

For the 6'-monophosphates, an additional product in low yield appeared with an R_F of 0.12.

For the main reaction products of cyclization, the ratio of P to molar extinction was 1, and they were also resistant to prostate monophosphatase (see below for conditions used). The cyclic phosphates were submitted to the action of 0.5 N-HCl at 100° in sealed ampoules; at given time intervals, ampoules were withdrawn, opened, and the contents chromatographed in solvent *B*. For all the monophosphate isomers, the main product of hydrolysis was monophosphate of *I*; in the case of the cyclization products of 2'- and 3'-monophosphates, several percent uracil made its appearance (checked chromatographically and spectrally). Complete hydrolysis was achieved after 5 hr. Hydrolysis of the cyclic phosphates in 0.1 N-HCl for 4 hr. resulted in the formation of 20 - 30% monophosphates.

The cyclohexylurea derivatives were transformed quantitatively to cyclic phosphates and monophosphates by exposure to pH 2 for 18 hr. at room temperature. This process was accompanied by the appearance of a water-insoluble precipitate which was soluble in ether and is undoubtedly dicyclohexylurea.

Treatment of 3 mg. quantities of the 2'-, 3'- and 6'-monophosphates of *I* with 0.5 ml. trifluoroacetic anhydride [4] at room temperature for 24 hr. gave, in each

case, a product in about 10% yield with an R_F of 0.25 in solvent *B*, and traces of a product with an R_F of 0.55.

When 3 mg. quantities of the foregoing isomeric monophosphates in 0.5 ml. dimethylformamide were treated with an excess of DCC (about 20 mg.) for 18 hr. at room temperature, the main reaction product, with an R_F in solvent *B* of 0.25, appeared in yields up to 20 - 25%. In addition, there were small yields of product with R_F 0.55 in solvent *B*, but no evidence of formation of phosphorylurea with R_F about 0.9.

1-(β-D-Glucopyranosyl)uracil-6′-pyrophosphate-glucose (VI). This compound was prepared according to the procedure described for UDPG by Roseman *et al.* [24]. Condensation of the 6′-phosphomorpholidate of *I* (0.09 m-mole) with the tri-*n*-octylamine salt of glucose-1-phosphate (0.5 m-mole) was carried out in anhydrous pyridine for 3 days at room temperature. The reaction products were fractionated on a 10 × 1 cm. Dowex 1 × 8 (Cl^-) column, unreacted morpholidate and 6′-phosphate of *I* being eluted with 0.02 M-LiCl and 0.003 N-HCl. The condensation product was then eluted with 0.06 M-LiCl and 0.003 N-HCl and isolated as the lithium salt by precipitation from methanol solution with acetone and ether. Yield was 62% theor.; the product exhibited only one spot in solvents *B* and *C*, exhibited the UV spectrum characteristic of *I* and had a ratio of P to molar extinction of 2. Heating in 0.01 N-HCl at 100° for 10 min. resulted in partial hydrolysis to the 6′-pyrophosphate of *I* and free glucose; in 1 N-HCl at 100° for 30 min., the products were the 6′-monophosphate of *I* and glucose. The course of hydrolysis was controlled by chromatography in solvents *B* and *C*, the products being revealed on the chromatograms by means of an UV lamp and by the aniline phthalate reaction [21].

Enzymic trials

These were conducted at a substrate concentration of 5 mg./ml., enzyme concentration of 0.5 mg./ml. and the appropriate buffer at a concentration of 0.1 M. An enzyme-free control was used for each experiment. Incubation was at 37° and aliquots were removed at given time intervals for chromatography.

Prostate monophosphatase [20]. Incubation in acetate buffer pH 5.2 with addition of Mg^{2+}.

(a) The 2′-, 3′- and 6′-phosphates of *I* were hydrolysed in 2 hr. quantitatively to *I*.

(b) 6′-Phosphate of *VII* was quantitatively converted to *VII* in 2 hr.

(c) Cyclization products of 2′-, 3′- and 6′-phosphates of *I* (with R_F 0.25 in solvent *B*) were unaffected even with prolonged incubation.

Kidney monophosphatase (Worthington). The 2′-, 3′- and 6′-monophosphates of *I*, and the 6′-monophosphate of *VII*, were all converted to the nucleosides after 5 hr. incubation.

Snake venom (Crotalus adamanteus). Incubation at pH 8.8 borate buffer in presence of Mg^{2+}.

(a) 6′-Phosphate of *I*, after 24 hr. incubation, exhibited 10% of *I*.

(b) 6′-Phosphate of *VII* was unaffected after 2 hr. incubation. After 24 hr. incubation, 60% was dephosphorylated to *VII*.

(c) 6′-Pyrophosphate of *I* was unaffected after prolonged incubation.

(d) Cyclization products of 2′-, 3′- and 6′-phosphates of *I* (with R_F 0.25 in solvent *B*) were unaffected even with prolonged incubation.

Pancreatic ribonuclease (Armour). Incubation at pH 7.2 in phosphate and in tris buffer. None of the cyclic phosphates (with R_F value of 0.25) was affected after 3 hr. incubation.

Polynucleotide phosphorylase (gift of Dr. Ochoa). Seven mg. of 6′-pyrophosphate of *I* incubated as described by Grunberg-Manago *et al.* [11]. After 48 hr. incubation only the unchanged compound could be detected.

Rabbit brain extract [6]. Rabbit brain was homogenized with 3 vol. 0.2 M aqueous saccharose and centrifuged for 15 min. at 20,000 *g* in a Serval centrifuge at 2°. The supernatant was brought to 40% saturation with ammonium sulfate at 0° and the resulting precipitate was dissolved in 5 ml. 0.02 M-tris buffer, pH 7.5. The solution was dialysed against 6 liter tris buffer, pH 7.5, for 20 hr. at 2° and then stored in a deep-freeze at -60°.

For enzymic tests, the system contained 0.1 ml. substrate (1 mg./ml.), 0.05 ml. 0.2 M-tris buffer, pH 7.5, 1 µl. of 0.1 M-$MgCl_2$, and 0.05 ml. brain extract containing 14 mg. protein/ml. [14]. Incubation was carried out at 37° and aliquots were removed for chromatography in solvent *B* after 45 min. and 20 hr. For the longer incubation times, both in this and other experiments described below, thymol was used to inhibit any bacterial growth which might have interfered with the estimations. The presence of thymol had no effect on enzymic activity.

(*a*) The cyclization products of 2′-, 3′- and 6′-phosphates of *I* all showed, after 45 min. incubation, several percent of monophosphates and no nucleosides. The isomeric monophosphates produced were not identified. Following 20 hr. incubation, 70% decyclization had taken place, 50% in the form of monophosphates and 20% nucleoside.

(*b*) One trial of the foregoing system was made, using as substrate uridine-2′:3′-phosphate. After 45 min. incubation, 70% was converted to uridine monophosphate. Following 20 hr. incubation all the cyclic phosphate had disappeared to give 20% monophosphate and 80% nucleoside.

(c) Glucose-4:6-cyclic phosphate, prepared as described by Khorana *et al.* [17] was incubated in the foregoing system, chromatographed in solvent *B*, the chromatograms being developed with aniline phthalate [21]. After 20 hr. incubation the products included 30% of the unchanged cyclic phosphate, 40% glucose-6-phosphate and 30% glucose.

(*d*) The various cyclic phosphates of *I* were incubated under identical conditions as above, with addition to the incubation medium of 20 µl. 0.1 M-sodium versenate. After 20 hr. incubation the products included 70 - 80% unchanged cyclic phosphates and 30 - 20% monophosphates.

(*e*) When uridine-2′:3′-phosphate was incubated as described in (*d*), the results were the same as those described in (*b*).

Calf liver extract. The extracts were prepared as described above for brain extracts. Incubation conditions were as follows: 100 µl. substrate (1 mg./ml.), 50 µl. 0.2 M-tris buffer pH 7.55, 1 µl. 0.1 M-$MgCl_2$ and 100 µl. extract with a protein content of 55 mg./ml.

(*a*) The cyclic phosphates of *I*, following incubation for 20 hr. at 37°, underwent 20% decyclization, the only product being glucosyluracil due to the monophosphate present in the extract.

(*b*) Following incubation of glucose-4:6-phosphate for 20 hr. at 37°, only 10% remained unchanged, the products being 80% glucose phosphate and 10% glucose.

We should like to thank Dr. W. Ostrowski for a gift of prostate phosphomonoesterase, Dr. S. Ochoa for a preparation of polynucleotide phosphorylase, and Mr. V. Jezdic for his assistance in the preparation of glucosylthymine.

SUMMARY

The synthesis is described of all four isomeric monophosphates of 1-(β-D--glucopyranosyl)uracil and of the 4′(6′)-monophosphates of 1-(β-D-glucopyranosyl)-thymine. Procedures are also outlined for fractionation of all the isomers on a Dowex column as well as for their identification. Also synthesized were the 6′-pyrophosphate of 1-(β-D-glucopyranosyl)uracil and a glucose analogue of UDPG, viz. 1-(β-D-glucopyranosyl)uracil-6′-pyrophosphate-glucose.

A study was made of the use of several procedures for preparation of the cyclic phosphates of the various monophosphates of glucosyluracil. All of these give products additional to those of the cyclic phosphates, although under identical conditions glucose-6-phosphate gives only one product, glucose-4:6-cyclic phosphate. Some of the properties of the glucopyranosyl-uracil cyclic phosphates are described.

An examination was made of the susceptibility to different enzymes of the various mono- and cyclic phosphates. The 6′-monophosphates of glucosyluracil and glucosylthymine are both dephosphorylated by snake venom, notwithstanding that glucose-6-phosphate is unaffected. Enzymes are also present in rabbit brain and in rat liver which hydrolyse all the cyclic phosphates to monophosphates; the enzyme(s) in rabbit brain are much more active than those in liver. Since glucose--4:6-phosphate is also transformed to the monophosphate by the same tissues, the specificity of the enzymes involved remains to be established. The fact that glucose-4:6-phosphate undergoes enzymic decyclization suggests that the enzyme(s) involved may play some role in intermediary metabolism.

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OTRZYMYWANIE ORAZ CHEMICZNE I ENZYMATYCZNE WŁAŚCIWOŚCI FOSFORANÓW 1-(β-D-GLIKOPYRANOZO)URACYLU I -TYMINY

Streszczenie

Przeprowadzono syntezę czterech izomerycznych monofosforanów 1-(β-D-glikopyranozo)uracylu i 4′(6′)-monofosforanów 1-(β-D-glikopyranozo)tyminy. Opisano warunki rozdziału wszystkich czterech izomerów na kolumnie Dowex, a także metody ich identyfikacji. Wydzielono i opisano produkty pośrednie syntezy tj. pochodne benzylidenowe, acetylowe i trójfenylometanowe nukleozydów, które stanowiły związki wyjściowe do selektywnej fosforylacji. Stwierdzono m.in. specyficzną migrację grupy acetylowej estryfikującej grupę wodorotlenową w pozycji 4′ w 1-(2′,3′,4′-trójacetylo-β-D-glikopyranozo)uracylu w kierunku pozycji 6′ pod wpływem kwasu octowego.

Przeprowadzono syntezę 6′-pirofosforanu 1-(β-D-glikopyranozo)uracylu i glikozowego analogu UDPG tj. P^{I}-6′[1-(β-D-glikopyranozo)uracyl]-P^{II}-1(α-D-glikozo)-pirofosforanu.

Przeprowadzono syntezy cyklicznych fosforanów pochodnych izomerycznych monofosforanów 1-(β-D-glikopyranozo)uracylu przy pomocy *N,N′*-dwucykloheksylokarbodwuimidu oraz bezwodnika kwasu trójfluorooctowego. We wszystkich wypadkach oprócz odpowiednich cyklicznych fosforanów otrzymano produkty uboczne; natomiast w identycznych warunkach cyklizacji 6-fosforan glikozy tworzył jako jedyny produkt reakcji 4:6-cykliczny fosforan glikozy. Opisano niektóre właściwości cyklicznych fosforanów 1-(β-D-glikopyranozo)uracylu i otrzymane wyniki porównano z danymi opisanymi w literaturze; m.in. stwierdzono powstawanie ureidopochodnych nukleotydów w czasie syntetyzowania 6- i 7-członowych cyklicznych fosforanów.

Zbadano podatność otrzymanych mono- i cyklicznych fosforanów na działanie niektórych nukleaz. 6′-Monofosforany glikozouracylu i glikozotyminy ulegają defosforylacji pod wpływem jadu węża, natomiast 6-fosforan glikozy nie ulega jego działaniu. Enzymy zawarte w ekstraktach z mózgu królika lub wątroby cielęcej powodują hydrolizę wszystkich otrzymanych nukleotydów cyklicznych do monofosforanów; enzymy mózgu królika są dużo bardziej aktywne niż enzymy wątroby cielęcej. Ponieważ powodują one także hydrolizę 4:6-fosforanu glikozy specyficzność ich pozostaje do ustalenia. Fakt, że 4:6-fosforan glikozy ulega enzymatycznej decyklizacji sugeruje, że enzymy te mogą odgrywać rolę w metabolizmie pośrednim.

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THE METHOD OF COMBINED COLUMN-PAPER CHROMATOGRAPHY APPLIED TO THE DETERMINATION OF AMINO ACIDS

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The unsatisfactory separation by paper or column chromatography is one of the major difficulties in the determination of amino acids in biological material. Haugaard & Kroner [5] applied the combination of electrophoresis with paper chromatography and at present this rather complicated technique is frequently used, particularly since the adoption of high-voltage electrophoresis. Using column chromatography followed by paper chromatography Boulanger, Biserte & Courtot [1] have analysed the composition of amino acids of urine, and Christensen *et al.* [2] as well as Lundquist & Galatius-Jensen [9] other body fluids. Such procedure, however, requires a troublesome placing of eluates collected with the fraction--collector on the paper.

To overcome this inconveniency a method [12] was elaborated for combined column-paper chromatography in a suitable apparatus [13] which directly applies the eluate from the column onto sheets of paper.

EXPERIMENTAL

Apparatus. Columns 0.8 × 45 cm. filled with starch. An appliance providing a concentration gradient of solvents (Fig. 1). An automatic device for placing the eluate from the column on separate sheets of paper, moving along continuously at the rate of 1 cm. per hour (Fig. 2).

Reagents and adsorbents. Ninhydrin 0.5% (w/v) in ethanol with additional 1 ml. of 1 N-NaOH per 100 ml. of the solution. Potato starch passing through a sieve of 0.25 mm. mesh, washed thoroughly with water and 50% ethanol. Ethanol 55% (v/v), and ethanol 25% (v/v) alkalized with ammonia (2.5 ml. of 20% ammonia per 100 ml.). Whatman no. 1 or no. 4 paper. Amberlite IR-120, Wofatite KPS-200, or any other sulphonated cation exchanger.

Procedure. A thick suspension of starch in 90% ethanol was poured into a column container placed vertically. As the starch settled down and ethanol flowed out, more suspension was added up to the point where the column reached the desirable

height (40 cm.). At the moment when the depth of ethanol above the starch was 1 cm., the column was connected with the container of solvent by means of a tube. The adsorbent was washed with 30 ml. of ethanol - ammonia mixture (90 ml. of 55% ethanol +10 ml. of 20% NH_3 solution) and with 55% ethanol, to neutral reaction. Then 0.5 - 3.0 mg. of a mixture of amino acids dissolved in 0.2 - 1.0 ml.

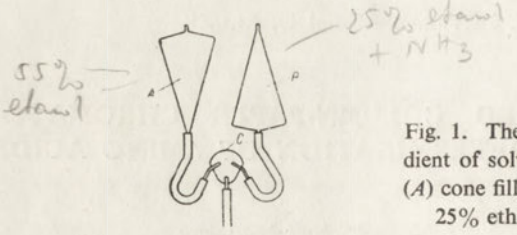

Fig. 1. The appliance for providing concentration gradient of solvents. Conical vessels of 50 ml. vol. each, (*A*) cone filled with 55% ethanol; (*B*) cone filled with 25% ethanol and ammonia; (*C*) mixing chamber

of 55% ethanol was applied. Immediately after the solution had soaked in, small portions of ethanol were added and the column connected with the mixing chamber. The cone placed base up contained 55% ethanol and another placed base down contained 25% ethanol with ammonia added.

In order to obtain a suitable concentration and pH of the solvent, the levels of liquid in the vessels were set in such a way as to allow first the 55% ethanol to start flowing out, to be joined after half an hour by the ethanol - ammonia solution.

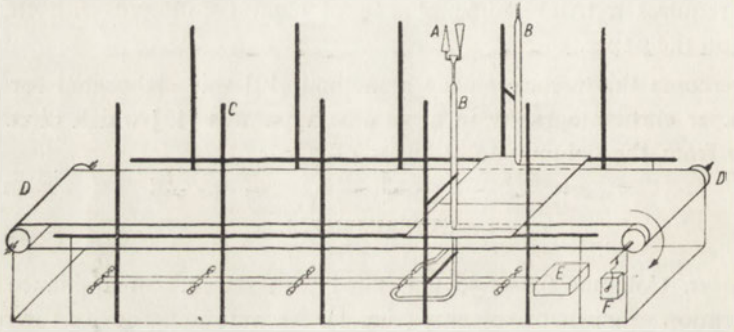

Fig. 2. Apparatus for column-paper chromatography. (*A*), Containers for solvents; (*B*), columns; (*C*), stand for fixing the columns; (*D*), two cylinders with cloth stretched between them; (*E*), blower provided with a heater and tubes bringing warm air under each column; (*F*), electric clockwork

Care had to be taken not to leave air bubbles in the tubes connecting the vessels to the column. The flow-rate of the eluate from the column was 16 ml. per day and could be controlled either by means of suitable packing of the starch or by connecting a capillary to the lower end of the column.

The eluate from the column was collected on separate sheets of paper along their longer sides, 10 cm. from the edge. The paper was carried on the cloth of the device (Fig. 2) at the rate of 1 cm. per hour. Warm air was passed under the sheet

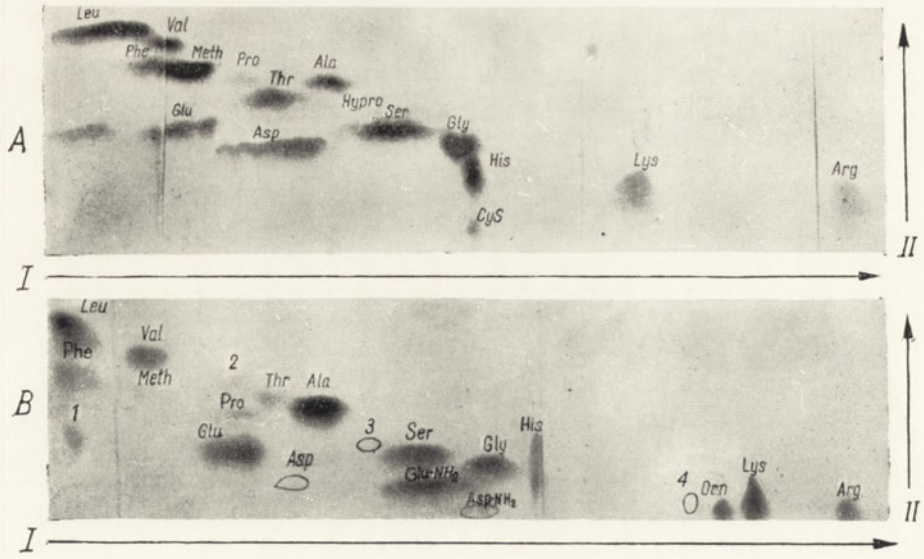

Fig. 3. Chromatogram of (*A*), standard mixture of amino acids; (*B*), human plasma amino acids. (*I*), Direction of placing the eluate from the column; (*II*), ascending chromatography. Abbreviations: Leu, leucine; Val, valine; Phe, phenylalanine; Meth, methionine; Pro, proline; Ala, alanine; Hypro, hydroxyproline; Ser, serine; Glu-NH_2, glutamine; Glu, glutamic acid; Gly, glycine; Asp-NH_2, asparagine; Asp, aspartic acid; His, histidine; CyS, cystine; Orn, ornithine; Lys, lysine; Arg, arginine; 1,2,3,4, non-identified spots

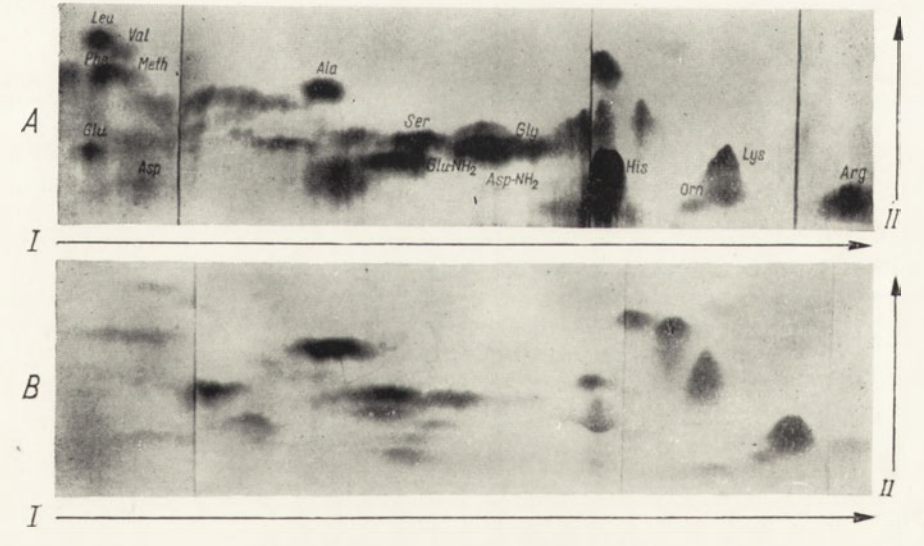

Fig. 4. Chromatograms of (*A*), amino acids from human urine; (*B*), amino acids from canine urine. Abbreviations as in Fig. 3.

where the solution was dropped. Each sheet was kept moving for 2 days and then was replaced by a new one. The whole eluate from one column was collected on 3 sheets of paper. Then ascending chromatography in 98% methanol was carried out and amino acids were detected by the method of Kay *et al.* [6].

Preparation of biological material. To 6 ml. of human serum an equal volume of absolute ethanol was added, the precipitated protein filtered off and washed twice with 50% ethanol. The combined filtrates were made up with water to 40 ml. and desalted by passing through Wofatite KPS-200, the ion-exchanger being previously transformed in acid form with 1 N-hydrochloric acid and washed with water to neutral reaction.

The solution of amino acids was run through a column of Wofatite 1.2 cm. in diameter and 14 cm. in height. Subsequently the column was washed with about 600 ml. of distilled water and the retained amino acids were then eluted with 50 ml. of 6 N-NH_3. The ammoniacal eluate was evaporated to dryness on a water bath, and the traces of ammonia removed by adding a small volume of distilled water and re-evaporating. The dry residue was dissolved in 1 ml. of 50% ethanol and transferred into a starch column. Samples of urine were prepared for chromatography in a similar way.

RESULTS

The analysis of 1 mg. of a standard mixture of amino acids or of protein hydrolysate gave chromatograms with a characteristic distribution of well-defined spots of particular compounds. The changes in ethanol concentration, in pH or in the rate of solvent flow through the column affected the localization of the spots. When, however, the procedure was exactly repeated, reproducible results were obtained from series of columns. Fig. 3A shows a chromatogram of a standard mixture of amino acids. On the chromatogram of human serum (Fig. 3B) besides the identified amino acids, spots ot other compounds (1,2,3 and 4) were present.

The chromatogram of 5 ml. of urine desalted on Amberlite IR-120 contained more ninhydrin-positive compounds. Beside amino acids, many other spots were found, a group of which with a high R_F, located at the right upper part of the chromatogram over histidine, attracts particular attention (Fig. 4). Preliminary attempts to identify them indicated that they were aminosugars and aminoalcohols.

Quantitative determination of amino acids

In order to plot the calibration curves for individual amino acids a series of standard mixtures containing 10 - 200 μg. of individual compounds was prepared and they were separated by means of the column-paper method and evaluated according to Kay *et al.* [6]. For that purpose each sheet of paper was sprayed with 0.5% ninhydrin solution and heated at 60° for 10 min. The spots were cut out, eluted with ethanol and determined on Pulfrich photometer with the Elpho attach-

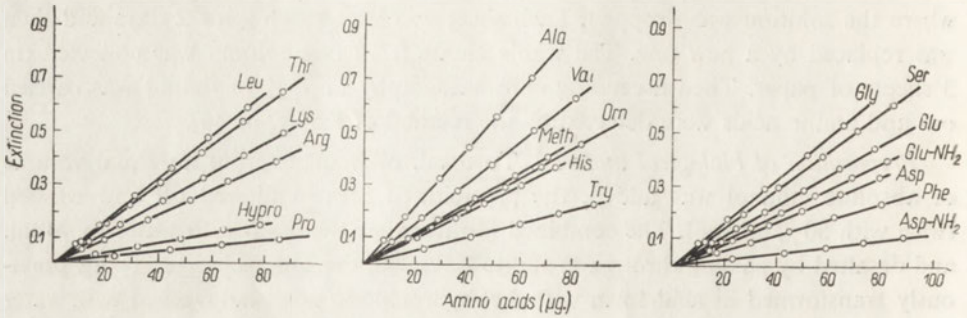

Fig. 5. Standard curves of amino acids, separated from standard mixture by column-paper chromatography, stained with ninhydrin and eluted

ment, using the S-53 filter, and for proline the S-42. Extinctions were read against the eluate from a piece of paper of the same size cut out in a place free from amino acids. The calibration curves are shown in Fig. 5.

Table 1

Losses in amino acids subjected to column-paper chromatography

Losses calculated in relation to the extinction of eluted spots without chromatography

Amino acid	Without chromatography		After chromatography		Losses (%)
	E	µg.	E	µg.	
Leucine	0.75	95	0.64	82	14
Valine	0.76	100	0.64	84	16
Phenylalanine	0.27	91	0.235	76	17
Methionine	0.56	97	0.45	78	20
Glutamic acid	0.57	96	0.52	88	9
Aspartic acid	0.44	100	0.36	83	17
Proline	0.105	90	0.088	75	17
Hydroxyproline	0.20	108	0.15	80	25
Threonine	0.89	121	0.70	96	21
Alanine	0.93	86	0.84	77	11
Tyrosine	0.21	90	0.195	83	8
Tryptophan	0.41	150	0.34	124	17
Serine	0.77	104	0.65	86	18
Glutamine	0.52	99	0.48	91	8
Glycine	0.75	92	0.72	88	4
Asparagine	0.115	92	0.095	81	12
Histidine	0.48	93	0.43	84	10
Ornithine	0.59	111	0.56	97	13
Lysine	0.56	101	0.54	97	4
Arginine	0.40	88	0.375	83	6

Estimation of the error and of losses in amino acids

In order to evaluate the losses of particular amino acids in the course of the column-paper chromatography, five successive analyses were carried out on the standard mixture. Ethanol solutions of individual amino acids were simultaneously prepared, each containing in 0.3 ml. an amount equal to that subjected to column--paper chromatography; three 0.3 ml. samples of each of these solutions were placed on the paper, then sprayed, eluted and determined as above, and averages taken as standard values. The mean values obtained after column-paper chromatography were referred to these standard values. The results of the two series of determinations are shown in Table 1.

Table 2

Estimation of the error of the method for determinations of amino acids subjected to column-paper chromatography

Mean values from 5 determinations are given. S. D.%, relative standard deviation $\left(\frac{100\,s}{\bar{x}}\right)$;

$$s = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}},$$

where x, value of an individual determination; $\bar{x}$, mean; n, number of determinations; s, standard deviation of an individual determination.

Amino acid	Taken (μg).	Found (μg.)	S.D. (%)
Leucine	64	62	4.9
Valine	66	64	4.1
Phenylalanine	60	57	6.3
Methionine	65	62	5.6
Glutamic acid	64	63	2.1
Aspartic acid	66	64.5	3.2
Proline	60	54	13.7
Hydroxyproline	72	68.5	7.3
Threonine	80	80	1.5
Alanine	58	57.5	1.9
Tyrosine	60	57	6.6
Tryptophan	100	98	5.3
Serine	70	71	1.4
Glutamine	66	64.5	4.7
Glycine	61	60	5,0
Asparagine	61	58	6.9
Histidine	72	69	4.2
Ornithine	74	72.5	3.1
Lysine	67	67.5	1.9
Arginine	59	57	4,3

In order to calculate the standard deviation for each amino acid, determinations of five succesive analyses of a mixture of known quantities of amino acids were carried out. Table 2 shows the relative standard deviation calculated in usual way.

DISCUSSION

A satisfactory separation of amino acids present in biological material is difficult to achieve because other amino compounds such as peptides, aminosugars and aminoalcohols are also present. The separation by paper chromatography as well as by column chromatography performed according to Moore, Spackman & Stein [10], and to Hannig [4] was sometimes unsatisfactory. Knauff & Schabert [7] reported that in the analysis of an artificial mixture or protein hydrolysate by the column method, a relatively good separation was obtained with the exception of serine, taurine and glutamine, but its application to body fluids, especially to urine, gave unsatisfactory results.

The quantitative determinations of individual spots are resolved more or less satisfactorily provided that a good separation of the components was obtained. Most frequently the analysis is performed by colorimetry of the eluted spots stained with ninhydrin. However, the obtained values do not account for the whole amount of amino acids present in the chromatographed material because of the losses depending on the kind of solvent used and the distance amino acids had travelled along the paper (Gerok [3], Kofranyi [8], Opieńska-Blauth [11]). These losses form an important obstacle in the determination, especially when the content of the analysed compound is relatively low. Maximum amount of the whole mixture which may be used in the two-dimensional chromatography is limited to 150 - 200 μg. The use of such small samples is one of the causes of important relative errors.

The presented column-paper chromatography enables the use of greater amounts facilitating the analysis of compounds present in small amounts. The simple construction of the apparatus for direct placing of the eluate from the columns on paper sheets enables the mounting of several columns and the carrying out of a series of analyses simultaneously. This extends the possibilities of performing serial analyses by means of various adsorbents and solvents.

The application of starch as adsorbent and ethanol of a concentration gradient from 55 to 25% as solvent was found very advantageous. The gradually increasing water content in the solvents and raising pH accelerated the outflow of amino acids of low R_F values and particularly of the basic ones. Under these conditions serine was separated from glycine, and ornithine, lysine and arginine were isolated as separate spots, which was difficult to achieve by the paper chromatography alone. The use of starch as adsorbent enabled the isolation from urine of a group of amino--compounds eluted from the column after histidine. In paper chromatography these amino-compounds overlap the neutral amino acids of high R_F values, even when various solvents are applied.

SUMMARY

A method and apparatus for combined column-paper chromatography for separation of amino acids was elaborated. The eluate from starch column flows out directly on the paper moved automatically. The method was found useful for the analysis of amino acids in blood and urine.

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METODA POŁĄCZONEJ CHROMATOGRAFII KOLUMNOWO-BIBUŁOWEJ W ZASTOSOWANIU DO OZNACZANIA AMINOKWASÓW

Streszczenie

Opracowano metodę i aparaturę do połączonej chromatografii kolumnowo-bibułowej dla rozdziału aminokwasów. Eluat z kolumny skrobiowej spływa bezpośrednio na automatycznie posuwającą się bibułę. Metoda okazała się przydatną do analizy aminokwasów we krwi i w moczu.

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L. ŻELEWSKI, Z. ALEKSANDROWICZ and C. DZIADUL

THE INFLUENCE OF OESTRADIOL BENZOATE AND NICOTINAMIDE ON CITRATE EXCRETION IN FEMALE RATS

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The results presented by several authors indicate that there is some connection of oestrogens with the amount of citrate in the urine [6, 8]. In our previous experiments [10] we have observed a decrease of urine citrate excretion in rats which had been given large doses of oestradiol benzoate; the fall was most marked on the fourth or fifth day after the injection. It was also reported [4] that oestrogens *in vitro* influence the NAD(P) transhydrogenase reaction. In the present study we have investigated the influence of oestradiol benzoate on the excretion of citrate and α-ketoacids in the urine of rats having a high level of pyridine nucleotides in the tissues. To obtain this level the oestradiol-treated rats were given nicotinamide which is known to cause an increase of pyridine nucleotides in the tissues [5].

MATERIALS AND METHODS

Albino female rats fed with the standard laboratory diet [9] were used for the experiments. In the first series 10 rats aged 6 months and weighing 160 - 220 g. were injected intramuscularly with oestradiol benzoate dissolved in arachis oil (10 mg. per kg. body weight) and intraperitoneally with nicotinamide (500 mg./kg.). The same dose of nicotinamide was repeated on the next day. The control group (10 rats) was treated in the same way with nicotinamide but instead of oestradiol received arachis oil alone.

In the second series 8 rats aged 4 months and weighing 180 - 220 g. were given oestradiol benzoate and nicotinamide in the same way as in the first series.

The third series consisted of 5 rats aged 4 months and weighing 160 - 190 g. They were given the same dose of nicotinamide and ten times lower dose of oestradiol benzoate (1 mg. per kg. body weight).

Chemical analysis. The examination of the urine was begun two days before the injections. The urine was collected to cylinders with 1 ml. of N-H_2SO_4. Citrate was estimated according to Beutler *et al.* [1], α-ketoacids with the method of Friedmann & Haugen [2], chloride with the method of Volhard [3], creatinine according

Table 1

The influence of coupled oestradiol benzoate and nicotinamide treatment on citrate excretion in the urine

Female rats were given 10 mg./kg. body wt. of oestradiol benzoate in arachis oil intramuscularly and 500 mg./kg. of nicotinamide intraperitoneally. On the next day the same dose of nicotinamide was repeated. The control rats were given arachis oil alone (without oestradiol) and nicotinamide. The day of oestradiol injection is marked with an asterisk. The values represent m-moles citrate per kg. body wt. per day, ± S. E. The daily amount of urine is expressed in ml. per kg. body wt., ± S. E. In parentheses the *p* values calculated from Student's *t* test are given.

Number of rats	Treatment	Excreted	Day of the experiment			
			1	2*	3	4
10	Arachis oil (control)	Citrate	0.36 ± 0.04	0.35 ± 0.04	0.38 ± 0.04 (0.8)	0.27 ± 0.06 (0.3)
		Urine	70 ± 7	62 ± 7	69 ± 6 (0.2)	69 ± 8 (0.2)
10	Oestradiol benzoate	Citrate	0.37 ± 0.03	0.34 ± 0.3	0.50 ± 0.06 (0.02)	0.05 ± 0.01 (0.001)
		Urine	57 ± 9	60 ± 8	64 ± 8 (0.3)	50 ± 7 (0.5)

Table 2

The influence of coupled oestradiol benzoate and nicotinamide treatment on the excretion of citrate, α-ketoacids, chlorides and nitrogen in the urine

Eight female rats were treated with oestradiol benzoate and nicotinamide as described in Table 1. The day of oestradiol injection is marked with an asterisk. The values represent m-moles of excreted compounds per kg. body wt. per day, ±S.E. In parentheses the *p* values calculated from Student's *t* test are given.

Compound	Day of the experiment					
	1	2*	3	4	5	6
Citric acid	0.52±0.04	0.49±0.03	0.76±0.07 (0.02)	0.18±0.03 (0.001)	0.08±0.02 (0.001)	0.29±0.02 (0.1)
α-Ketoacids	0.24±0.02	0.25±0.02	0.49±0.06 (0.01)	0.12±0.02 (0.001)	0.10±0.01 (0.001)	0.21±0.03 (0.8)
Chloride	13.6±1.3	15.6±0.9	7.3±0.8 (0.001)	6.7±1.1 (0.001)	9.8±1.2 (0.01)	7.4±1.7 (0.05)
Total nitrogen	121±6.6	116±10.7	111±7.2 (0.5)	85±7.2 (0.02)	78±9.3 (0.01)	74±14.3 (0.01)
Urine	57±5	70±7	112±13 (0.01)	91±17 (0.2)	108±16 (0.02)	112±12 (0.3)

to Benedict *et al.* [7] and calcium according to Kramer & Tisdall [7]. Total nitrogen of the urine was estimated with the Kjeldahl procedure in the Parnas-Wagner apparatus, distilling to the boric acid.

Statistical evaluation of the results has been made with the Student's *t* test, as described previously [10].

Reagents. Oestradiol benzoate was a gift of Hoechst A.G., Frankfurt am Main. 2,4-Dinitrophenylhydrazine was Light & Co. product. Other reagents were purchased from Fabryka Odczynników Chemicznych, Gliwice.

RESULTS

On the first day after the injection of oestradiol benzoate (10 mg./kg. body weight) and nicotinamide (500 mg./kg.) there was an increase of citrate excretion whereas on the second day a marked fall was observed (Table 1). This decrease can not be explained by any decrease of diuresis.

Table 3

The influence of coupled oestradiol benzoate and nicotinamide treatment on the excretion of citrate, α-ketoacids, chloride, nitrogen, creatinine, calcium and the amount of urine

Five female rats were treated with 1 mg./kg. body wt. of oestradiol benzoate and with nicotinamide as indicated in Table 1. The day of oestradiol injection is marked with an asterisk. The amounts of excreted compounds are expressed in m-moles per kg. body wt. per day, ±S.E. The daily volume of urine is presented in ml. per kg. body wt., ±S.E. In parentheses the *p* values calculated from Student's *t* test are given.

Compound	Day of the experiment					
	1	2*	3	4	5	6
Citric acid	0.51±0.01	0.62±0.01	0.82±0.01 (0.1)	0.25±0.01 (0.05)	0.36±0.04 (0.2)	0.43±0.02 (0.5)
α-Ketoacids	0.34±0.08	0.35±0.07	0.54±0.11 (0.02)	0.20±0.01 (0.1)	0.24±0.01 (0.4)	0.48±0.02 (0.3)
Chloride	14.9±3.2	15.5±2.3	10.7±1.8 (0.02)	12.5±2.3 (0.3)	15.2±2.3 (0.7)	14.1±1.6 (0.9)
Total nitrogen	103±15.7	101±15.7	111±15.0 (0.8)	117±10.0 (0.7)	110±10.0 (0.4)	103±5.0 (0.8)
Creatinine	0.31±0.04	0.30±0.04	0.34±0.04 (0.3)	0.34±0.03 (0.4)	0.37±0.04 (0.2)	0.34±0.02 (0.6)
Calcium	0.48±0.11	0.48±0.12	0.52±0.12 (0.4)	0.57±0.11 (0.2)	0.52±0.06 (0.3)	0.45±0.07 (0.9)
Urine	47±7	44±8	48±9 (0.01)	53±8 (0.2)	65±9 (0.02)	64±7 (0.05)

In the second series (Table 2) in which the analysis of urine has been prolonged to four days after the injection, the same changes of citrate excretion were observed. The greatest decrease was observed on the third day, and an increase was begun on the fourth day. The changes in excretion of α-ketoacids were similar to those of citrate. The amount of chlorides excreted in the urine decreased already on the first day after the injection and was low throughout the experiment. The daily excretion of total nitrogen was not changed on the first day after the injection but decreased permanently from the second to the fourth day. The diuresis was slightly increased.

When the rats were treated with ten times lower doses of oestradiol benzoate and the same doses of nicotinamide the changes in citrate and α-ketoacids excretion were the same as in the previous experiments, although the greatest decrease appearing on the second day after the injection was less pronounced (Table 3). The excretion of chlorides decreased only on the first and second day after the injection. The excretion of total nitrogen, creatinine and calcium did not change significantly during the whole period of experiment. Also in this experiment the diuresis was somewhat increased after oestradiol and nicotinamide injections.

DISCUSSION

It has been previously observed [10] that the greatest fall of citrate excretion in the urine appeared on the fourth or fifth day after the rats had been treated with oestradiol benzoate; the treatment consisted of two doses, 10 mg. per kg. body weight each, given every other day. The effect was modified when presently oestradiol benzoate was applied in a single dose coupled with nicotinamide. On the first day after the injection an increase of citrate excretion was observed, followed by a decrease already on the second and third day. The increase and decrease of citrate excretion were accompanied by the same changes of α-ketoacids excretion. It seems probable that the excretion of citrate and α-ketoacids is directly related. This is not the case with chlorides. Large doses of oestradiol benzoate caused already on the second day a decrease of chloride excretion which lasted till the fourth day of observations. The changes in total nitrogen excretion were also different from citrate and α-ketoacids. On the first day after the injection, when the excretion of citrate and α-ketoacids increased, the amount of total nitrogen in the urine was unchanged, and since the second day a permanent decrease was observed. It lasted till at least the fourth day, whereas at that time the excretion of citrate and α-ketoacids increased again. It may be that this resulted from a lower food intake after the injection.

The influence of coupled administration of oestradiol benzoate and nicotinamide seems to be specific for urine citrate and α-ketoacids excretion. This view is supported by the results obtained when ten times smaller doses of oestradiol benzoate had been given. In this case an increase of citrate and α-ketoacids excretion could also be observed on the first day, followed by a decrease on the second day. The amount of chloride excreted was lower only on the first and second day after the injection

and total nitrogen and creatinine underwent no significant changes throughout the experiment. Also the amount of calcium in the urine did not change which shows that there is no correlation of calcium and citrate excretion.

The results indicate that the effect of coupled oestradiol benzoate and nicotinamide treatment is specific for citrate and α-ketoacids excretion. It seems possible that oestradiol may influence the excretion of citrate and α-ketoacids by modifying the NAD(P) transhydrogenase reaction in the female organism.

SUMMARY

The coupled oestradiol benzoate (10 mg./kg. or 1 mg./kg. body weight) and nicotinamide (500 mg./kg.) treatment causes an increase of citrate and α-ketoacids excretion on the first day after the injection followed by a decrease on the second and third day.

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WYDALANIE CYTRYNIANU Z MOCZEM SAMIC SZCZURÓW PO PODANIU BENZOESANU ESTRADIOLU I NIKOTYNAMIDU

Streszczenie

Skojarzone podanie benzoesanu estradiolu (10 mg./kg. albo 1 mg./kg. wagi) z nikotynamidem (500 mg./kg.) wywołuje wzrost wydalania cytrynianu i α-ketokwasów w pierwszym dniu po injekcji, po czym w drugim i trzecim dniu następuje spadek ich wydalania.

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EFFECT OF MALEIC ACID ON THE KIDNEY

I. OXIDATION OF KREBS CYCLE INTERMEDIATES BY VARIOUS TISSUES OF MALEATE INTOXICATED RATS

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Experimental results *in vivo* (see ref. [1]) indicate that in the intoxication with maleate there appears a selective damage of kidney function. So far the mechanism of the toxic action of maleate on the kidney is little known. It has been shown *in vitro* that the system of oxidative decarboxylation of α-ketoglutarate is about 100 times more sensitive to maleate in kidney than in liver. Therefore the damage of the kidney was supposed to be connected with the inhibition of its oxidative decarboxylation system. In the present study it was found, however, that in brain and heart the inhibition of ketoglutarate oxidation *in vitro* is equal to, or even greater than in kidney; on the other hand, in rats treated with toxic doses of maleate, the inhibition of Krebs cycle was observed only in the kidney.

EXPERIMENTAL

Materials and methods. White Wistar rats were killed by decapitation. The brain, heart, kidneys and liver were removed as soon as possible and cooled in isotonic KCl solution or in 0.25 M-saccharose. Then the tissues were homogenized in a glass homogenizer with 2 volumes of 0.15 M-KCl or 0.25 M-saccharose in 10^{-3} M-EDTA. Mitochondria were obtained according to Hogeboom [3].

The maleic acid solution adjusted to pH 7.2 was injected intraperitoneally in a dose of 100 to 500 mg. per kg. body weight. The treated animals were killed 15 min., 3, 6 or 36 hr. after the injection.

The oxygen consumption was measured in a Warburg apparatus in the atmosphere of air using flasks with gas volume of about 15 ml.

Maleic acid was USSR product; α-ketoglutaric acid, CalBiochem.; citric, malic, succinic, glutamic and pyruvic acids were commercial preparations (Biuro Odczynników Chemicznych, Gliwice, Poland). Potassium salt of pyruvic acid was prepared after purification according to Lardy [5].

The effect of maleate in vitro *on the oxidation of ketoglutarate by homogenates of rat tissues*

It has been previously shown [8] that 5×10^{-4} M-maleate inhibited in 50% the consumption of ketoglutarate in kidney homogenates but had no effect on the liver. In experiments on the oxidation of ketoglutarate in various rat tissues it was found (Table 1) that at 2×10^{-3} M-concentration maleate inhibited oxygen

Table 1

Effect of maleate on the oxidation of ketoglutarate in rat tissues

Each Warburg vessel contained: 1 ml. homogenate corresponding to 330 mg. of wet wt. of tissues, 60 μmoles ketoglutarate, 50 μmoles of tris buffer, pH 7.2, 20 μmoles phosphate buffer, pH 7.2, 12 μmoles $MgCl_2$, 6 μmoles ATP, 1.5 μmole EDTA, 300 μmoles sucrose, and maleate as indicated in the Table. Final volume 3 ml. The oxygen uptake was measured at 30° for 60 min. in the atmosphere of air. The results show the oxygen uptake in μg.-atoms per 330 mg. of wet wt. per hr. Mean values from 6 - 7 experiments are given; figures in parentheses show the range.

Maleate conc. (M)	Kidney		Heart		Brain		Liver
	Oxygen (μg.-atoms)	Inhibition (%)	Oxygen (μg.-atoms)	Inhibition (%)	Oxygen (μg.-atoms)	Inhibition (%)	Oxygen (μg.-atoms)
None	31.5 (29.4—34.2)	—	43.5 (41.5—44.6)	—	48.5 (47.2—49.9)	—	49.2 (47.8—50.9)
5×10^{-4}	23.1 (20.1—24.4)	27	30.9 (29.4—31.5)	29	27.4 (26.9—28.5)	40	50.6 (49.1—52.3)
1×10^{-3}	19.5 (17.5—20.2)	38	24.4 (24.1—24.8)	44	15.0 (14.5—15.9)	69	44.5 (44.0—46.1)
2×10^{-3}	16.0 (14.5—17.2)	49	14.8 (13.9—14.9)	66	9.4 (8.9—9.8)	80	44.0 (43.7—46.1)
6×10^{-3}	12.0 (10.6—12.8)	62	10.3 (10.1—10.4)	76	—	—	51.1 (49.2—52.5)

consumption not only in kidney but also in brain and heart homogenates, and therefore the selective action of maleate on the kidney cannot be postulated. The liver appeared to differ from other tissues in that it was completely insensitive to maleate.

Oxidation of Krebs cycle intermediates by homogenates of tissues of maleate--intoxicated rats

The selective action of maleate on the kidney, which was not confirmed in the experiments *in vitro*, was supposed to take place *in vivo*. When maleate was administered to rats by intraperitoneal injections, it did accumulate in kidneys, the concentrations attained being 11 times greater than in liver and 7 times than in heart

(Angielski & Rogulski, unpublished data). It should be also pointed out that in experiments *in vitro* the tissues were treated with maleate after previous destruction of their cellular structure, which is certainly of great importance for the action of maleate *in vivo*.

The results shown in Table 2 confirmed our supposition. The homogenates of liver, heart, and brain, of maleate-treated rats oxidized all the investigated Krebs cycle intermediates to the same extent as homogenates of tissues of the control rats, whereas in kidney homogenates of intoxicated animals a marked inhibition of oxidation of all investigated substrates except succinate was observed. These results indicate that in maleate-treated rats there appeared a selective inhibition of the Krebs cycle confined to the kidney alone. These discrepancies with the experiments *in vitro* can be explained, at least in part, by differences in the permeability of cellular and mitochondrial membranes in particular organs. This is in agreement with Weil-Malherbe [10] who showed that oxidation of pyruvate in the presence of maleate was more inhibited in brain homogenates than in slices of brain.

Oxidation of ketoglutarate by rat tissues at different intervals after the injection of maleate

Maleate-treated rats were killed, and oxidation of ketoglutarate by their tissues was determined (Table 3). In the kidney already 15 min. after the injection a 63% inhibition was observed, which 3 hr. after the injection reached 75%; 20% inhibition

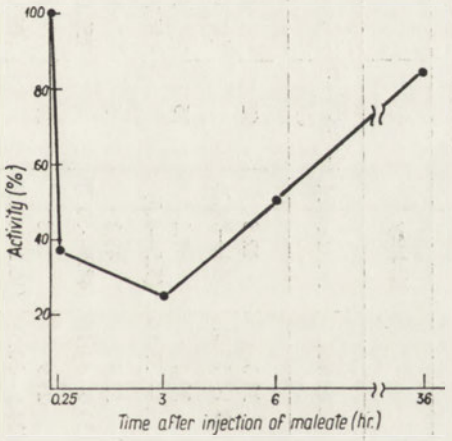

Fig. 1. The oxidation of α-ketoglutarate by rat kidney homogenates at different intervals after the injection of maleate. Conditions of experiment are described in Table 3

still persisted after 36 hr. (Fig. 1). It is worth mentioning that the decrease in SH groups in kidney [7] had a similar time-course. In brain, heart and liver no inhibition was observed.

Table 2

The oxidation of Krebs cycle intermediates in rat tissues homogenates 3 hr. after the injection of maleate

Maleate was injected intraperitoneally in a dose of 300 mg./kg. body weight. The conditions of incubation as in Table 1 but instead of sucrose 175 μmoles of KCl was given. Mean values from 6 experiments are given; figures in parentheses show the range. Results are expressed in μg.-atoms of oxygen per 330 mg. of tissue wt. per hour.

Substrate	Kidney			Heart		Liver		Brain	
	Control	Intoxicated	Inhibition (%)	Control	Intoxicated	Control	Intoxicated	Control	Intoxicated
Citrate	33.3 (30.1—35.0)	12.8 (10.1—14.2)	62	48.4 (45.9—50.3)	48.3 (46.8—51.7)	41.4 (38.7—43.2)	43.5 (40.0—45.2)	28.7 (25.0—30.1)	26.1 (24.2—28.5)
Ketoglutarate	30.4 (26.4—33.1)	7.8 (5.5—9.0)	75	51.8 (46.7—52.0)	52.3 (48.8—53.4)	51.0 (48.7—53.8)	50.5 (48.7—51.8)	42.7 (39.8—43.6)	43.1 (41.8—46.2)
Succinate	52.7 (50.0—54.1)	49.3 (48.5—50.7)	5	56.1 (53.1—58.4)	60.1 (58.0—61.2)	49.6 (47.5—52.1)	47.8 (45.6—49.7)	45.2 (43.1—48.2)	47.3 (45.3—50.6)
Malate	29.9 (28.5—30.5)	12.8 (10.2—13.5)	57	48.0 (46.3—50.0)	50.8 (46.9—52.9)	44.9 (41.8—46.4)	49.1 (46.1—52.5)	26.9 (23.8—30.0)	24.5 (22.1—27.3)
Pyruvate	28.0 (27.1—29.0)	10.9 (8.5—12.3)	61	49.3 (47.1—52.3)	47.9 (45.5—50.8)	45.8 (42.8—47.4)	47.9 (45.0—50.6)	39.3 (36.6—42.0)	41.1 (38.1—43.3)
Glutamate	35.8 (34.5—36.0)	14.5 (13.7—14.9)	60	49.7 (46.9—52.9)	46.8 (44.9—49.2)	49.0 (46.2—52.7)	45.8 (43.6—48.6)	39.6 (36.8—42.1)	38.3 (35.8—40.4)

Effect of the dose of maleate on the inhibition of ketoglutarate oxidation in rat kidney

Our previous experiments and the data of Niemiro [6] indicated that to produce a definite tubular deficiency of the kidney a dose of maleate over 250 mg. per kg. body weight was necessary. It was shown that the toxic dose of maleate is several times greater than the toxic doses of *N*-ethylmaleimide and of many other inhibitors of SH groups. Taggart, Angielski & Morell [9] have shown that maleic acid was metabolized by the kidney, and this explains the relatively small toxicity of this compound. The percentage of metabolized maleate was smaller when the injected dose was greater.

Table 4 presents the influence of the dose of maleate on the inhibition of ketoglutarate oxidation in rat kidney. Three hours after the injection of 200 mg. maleate per kg. body weight no effect on the oxidation of ketoglutarate was observed, whereas the dose of 300 mg. per kg. (used also in experiments described in Tables 2 and 3) resulted in 75% inhibition. At 500 mg. per kg. the inhibition of ketoglutarate oxidation was over 80%.

Effect of maleate on the oxidation of Krebs cycle intermediates by rat kidney mitochondria

For more detailed studies of the oxidation of Krebs cycle intermediates in the presence of maleate (Fig. 2) the kidney mitochondria prepared from saccharose were used. The greatest inhibiting effect was found for ketoglutarate and glutamate oxidation. At maleate concentration of 4×10^{-4} M, nearly 70% inhibition of oxygen consumption was observed. Citrate, malate and pyruvate exhibited much smaller sensitivity to maleate. Oxygen consumption in the presence of these substrates became definitely inhibited only at maleate concentrations nearly 10 times greater than in the case of ketoglutarate. Even very high maleate concentrations, 5×10^{-3} M, did not inhibit the oxygen consumption in the presence of succinate as a substrate, in agreement with the results of Hopkins *et al.* [4].

Maleate at 10^{-2} M concentration had no influence on ketoglutarate formation from citrate (Rogulski & Angielski, in preparation). Therefore the inhibition of oxygen uptake with citrate as a substrate may result from blocking of further oxidation of ketoglutarate. If this is correct, the inhibition should be independent from maleate concentration above the concentration which is blocking the oxidation of ketoglutarate. The obtained results were, however, quite different; 20% of inhibition of citrate oxidation was observed at the concentration of maleate which inhibited ketoglutarate oxidation in 76%. At the same time the inhibition of oxygen uptake was further dependent on maleate concentration and reached 70% at a concentration of 3×10^{-3}. It should be remembered that the formation of ketoglutarate from citrate, as mentioned above, is not affected by this maleate concentration.

Table 3

Oxidation of ketoglutarate by rat tissues homogenates at different intervals after the administration of maleate

Maleate was injected intraperitoneally in a dose of 300 mg. per kg. body wt. Non intoxicated rats were used as controls. Conditions as in Table 1. Mean values from 6 experiments are given; figures in parentheses show the range.

Time between the injection of maleate and the killing of animal	Kidney		Heart	Brain	Liver
	Oxygen (μg.-atoms)	Inhibition (%)	Oxygen (μg.-atoms)	Oxygen (μg.-atoms)	Oxygen (μg.-atoms)
Control	30.4 (26.4—33.1)	—	42.7 (38.9—45.3)	51.8 (48.7—53.4)	51.0 (49.2—53.4)
15 min.	11.3 (8.9—12.4)	63	42.3 (39.4—45.6)	52.4 (50.4—54.8)	51.3 (50.6—54.0)
3 hr.	7.8 (7.1—8.2)	75	43.1 (41.2—45.8)	52.3 (51.0—55.6)	50.5 (48.2—53.5)
6 hr.	15.6 (13.4—16.4)	49	43.4 (42.1—45.2)	51.2 (49.3—56.4)	54.2 (50.8—55.7)
36 hr.	25.0 (24.5—28.3)	18	—	50.5 (47.9—54.1)	—

Table 4

Inhibition of the oxidation of ketoglutarate by kidney homogenates in relation to the injected dose of maleate

Conditions as in Table 2. Maleate was given intraperitoneally and rats were killed 3 hr. after the administration of maleate. Mean values from 4 experiments are given; figures in parentheses show the range.

Dose of maleate (mg./kg. body weight)	Oxygen uptake (μg.-atoms/330 mg. of wet wt. of tissues/hr.)	Inhibition (%)
None	30.4 (26.4—33.1)	—
100	29.6 (29.5—30.1)	0
200	30.3 (29.8—31.2)	0
300	7.6 (7.0—8.4)	75
500	5.5 (4.9—6.0)	82

Also the inhibition of oxygen uptake in the presence of malate as a substrate was rather unexpected. From the data of Green [2] and Angielski & Rogulski (unpublished data) it follows that the activity of the purified preparation of malic acid dehydrogenase was not affected by 5×10^{-3} M-maleate, whereas in mitochondria at this concentration there was about 80% inhibition of oxygen consumption. Similarly as in the case of citrate, the enzyme was insensitive to maleate, but

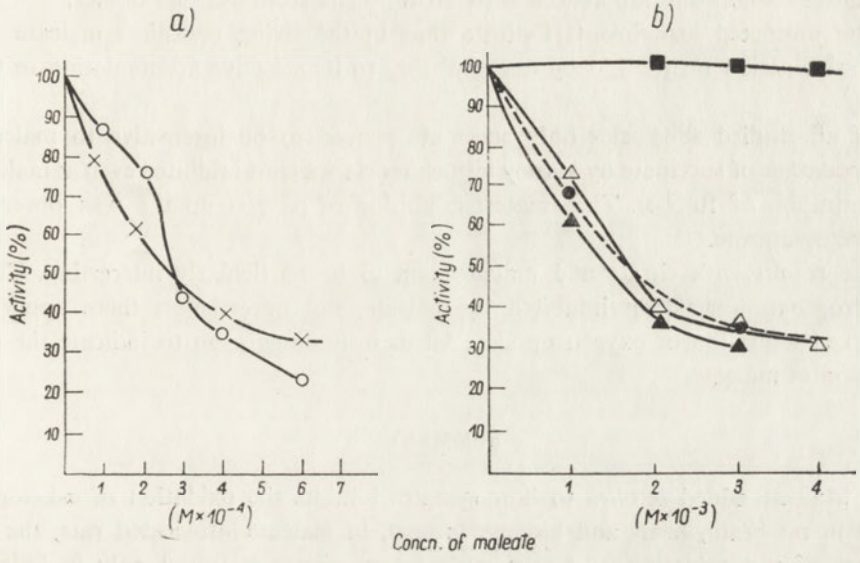

Fig. 2. Effect of maleate on the oxidation of Krebs cycle intermediates by rat kidney mitochondria. Mitochondria were preincubated with potassium maleate, pH 7.2, in 0.25 M-sucrose with 0.001 M-EDTA for 10 min. in an ice bath. The incubation mixture contained: 30 μmoles of substrate, 50 μmoles of tris buffer, pH 7.2, 20 μmoles phosphate buffer, pH 7.2, 12 μmoles $MgCl_2$, 6 μmoles of ATP, and 0.5 ml. of mitochondria (corresponding to 330 mg. of tissue wt.) preincubated with maleate. Final volume 2 ml. Oxygen uptake was measured in air for 30 min. at 30°. (*a*) o, Ketoglutarate, ×, glutamate; (*b*), ▲, malate, ●, pyruvate, △, citrate, ■, succinate

nevertheless an inhibition of oxygen uptake was apparent. These discrepancies can be explained if we assume that maleate inhibits the transfer of electrons via NAD. This may also explain the fact that succinate oxidation is unaffected by maleate.

It was shown [8] in an indirect way that glutamic acid dehydrogenase is insensitive to maleate. The inhibition of glutamate oxidation by maleate may be explained by assuming that in fact we are dealing with the inhibition of ketoglutarate arising from glutamate by transamination. This mechanism seems to explain satisfactorily the same sensitivity toward maleate of glutamate and ketoglutarate oxidation.

DISCUSSION

The biochemical differences between various tissues and organs are closely connected with differences in morphological structure of the cells and cellular organelles. The selectivity of tissues in regard to various drugs and poisons is probably determined by such differences, especially by the permeability of the membranes of cells and of mitochondria. The damage to cell structure and to compartmentation reduces the differences between the cells from various tissues.

The presented experiments indicate that in the living organism maleate has a selective action on the kidney due probably to its selective accumulation in this organ.

Of all studied substrates only succinate proved to be insensitive to maleate. The oxidation of succinate by kidney mitochondria was not inhibited even at maleate concentration of 10^{-2} M. The greatest inhibition of oxygen uptake was observed with ketoglutarate.

The results with citrate and malate seem to be particularly interesting. Their dehydrogenation was not inhibited by maleate, but nevertheless there appeared a marked inhibition of oxygen uptake. All these findings seem to indicate the site of action of maleate.

SUMMARY

1. Maleate added *in vitro* to homogenates inhibits the oxidation of α-ketoglutarate in rat brain, heart and kidney. *In vivo*, in maleate-intoxicated rats, the inhibition of the oxidation of Krebs cycle intermediates is found only in kidney.

2. The inhibition of α-ketoglutarate oxidation occurs only when big doses of maleate are given (300 mg./kg. body wt.).

3. *In vitro* the oxidation of α-ketoglutarate by kidney mitochondria is inhibited by maleate at concn. $4 - 5 \times 10^{-4}$ M, the oxidation of citrate, malate and pyruvate at concn. 6 times greater, and the oxidation of succinate is not affected even by 10^{-2} M concn.

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DZIAŁANIE KWASU MALEINOWEGO NA NERKĘ

I. UTLENIANIE ZWIĄZKÓW POŚREDNICH CYKLU KREBSA W TKANKACH SZCZURÓW ZATRUTYCH MALEINIANEM

Streszczenie

1. Maleinian dodany do homogenatu *in vitro* hamuje spalanie ketoglutaranu w mózgu, sercu i nerce. Natomiast *in vivo*, tzn. u szczurów zatrutych maleinianem, zahamowanie spalania związków pośrednich cyklu Krebsa występuje tylko w nerce. W 15 min. po zatruciu wynosi ono około 60%, po 3 godz. osiąga 75%, a jeszcze w 36 godz. od wstrzyknięcia maleinianu stwierdza się około 20% zahamowania.

2. Wyraźne zahamowanie spalania α-ketoglutaranu występuje tylko po podaniu szczurom dużej dawki maleinianu (300 mg./100 g. wagi).

3. Spalanie α-ketoglutaranu *in vitro* przez mitochondria nerek zostaje zahamowane w około 70% przy stężeniu maleinianu $4 - 5 \times 10^{-4}$ M, natomiast spalanie cytrynianu, jabłczanu i pirogronianu przy stężeniu 6 - 7 razy większym. Maleinian nawet w dużym stężeniu nie ma wpływu na spalanie bursztynianu.

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ST. GOLEWSKI

PHOSPHORYLATING ENZYMES OF NUCLEIC ACIDS

I. INCORPORATION OF [^{32}P]ORTHOPHOSPHATE INTO THYMUS NUCLEIC ACIDS

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The mechanism of incorporation of different compounds into nucleic acids, an essential feature of metabolism of living cells, is being investigated in many laboratories [see ref. 3]. In the present work the conditions of the incorporation of [^{32}P]orthophosphate into nucleic acids were studied.

MATERIALS AND METHODS

Calf thymus homogenates were used for the experiments. The homogenization of the tissue in a Waring-Blendor type apparatus, and the isolation of the enzymic fraction were carried out at 4° in a cold-room. The enzymic preparations were kept in test-tubes placed in a vacuum flask filled with ice.

Two kinds of experiments were performed: 1. The labelled [^{32}P]orthophosphate solution, in the amount corresponding to $4 \times 10^6 - 2 \times 10^7$ counts/min., was added to the tissue homogenate buffered with 0.01 M-tris solution, pH 7. Following incubation the nucleic acids were isolated and the specific activities of ribonucleic acid (RNA), in some cases of deoxyribonucleic acid (DNA), as well as the activities of the products of hydrolysis were examined. 2. The tissue homogenate was treated with 0.01 M-tris solution, pH 7, and centrifuged. The supernatant was assumed to contain a system which should catalyse the incorporation of [^{32}P]-orthophosphate into nucleic acids. This fraction is called further the enzyme or enzymic fraction. The nucleic acids present in the residue were then treated with enzyme and [^{32}P]orthophosphate, and incubated. Then the radioactive nucleic acids were isolated and their specific activity determined.

Proteins in enzymic solutions were determined according to Gornall *et al.* [7] colorimetrically (Lumetron, model 401 A) or spectrophotometrically (Unicam SP 500), their amounts being calculated according to Christian & Warburg [4]. The concentrations of nucleic acids solutions were estimated at 260 mμ and expressed in μmoles of nucleic phosphorus (P_{NA}) per 1 ml. of the solution. The

concentrations of non-hydrolysed nucleic acids were calculated using spectrophotometrical data and taking molar extinction (ε) as equal to 8. For the hydrolysate $\varepsilon = 10$ and for the separated ribonucleotides the ε values given by Cohn [2] were used. pH of the employed solutions was determined with a Ridan PCR type, model F pH-meter (Poland). Chromatography was carried out on Whatman no. 1 paper using Krebs & Hems [9] solvent system, and paper electrophoresis of the ribonucleotides was performed in a vertical apparatus and under conditions described elsewhere [6].

Mineralight model V 43 was used for the localization of spots on the filter paper. Radioactivity measurements were performed on 0.5 ml. samples of the examined solution with a G-M monitor type 20/58 (Poland) and General Scaler Ratemeter model RCR 2 (U.S.A.) fitted with an end-window G-M counter, type BAT 1 (1.34 mg./cm.^{2}).

The radioisotope, [^{32}P]orthophosphate, specific activity about 2 mc./ml., was purchased from the Institute of Nuclear Research, Warszawa, in the form of either H_3PO_4 or Na_2HPO_4 solutions which were hydrolysed before use in 1 N-HCl for 10 min. at 100° to decompose pyrophosphates, and then adjusted to pH 7 with 4 N-KOH.

Nucleic acids were isolated from the tissue according to Davidson & Smellie [5]. Since nucleic acids obtained with this method are partially depolimerized by high temperature, a number of experiments were performed with nucleic acids isolated under somewhat milder conditions according to Mirsky & Pollister [10]. This method permits, at the same time, to separate RNA from DNA taking advantage of the different solubilities of these compounds in 0.14 M-NaCl solution.

EXPERIMENTAL

Effect of the incubation time. Six samples, each containing 2 g. of thymus homogenate, 2 ml. of 0.01 M-tris buffer, pH 7.3, and [^{32}P]orthophosphate (corresponding to 4×10^6 counts/min.) were incubated at 37° for 0, 2, 4, 6, 8 and 12 hr., respectively, and then treated with 40% trichloroacetic acid (TCA). The precipitates were washed with 4 ml. of cold 10% TCA with non-radioactive phosphate added (0.01 M) for better washing out of the isotope. The precipitates were then suspended in 4 ml. of cold distilled water, adjusted with 1 N-NH_4OH to pH 4 (Lachema indicator) and centrifuged. Nucleic acids were extracted twice from the precipitates with 10% NaCl solution at 100° for 1 hr., using each time 2 ml. of NaCl solution [5]. The combined extracts were centrifuged, the supernatants treated with double volume of ethanol and left overnight at 0°. Then the centrifuged precipitates were dissolved in 0.01 M-tris buffer, pH 7.3, using 2 ml. of buffer solution for each sample. The concentration of nucleic acids was determined spectrophotometrically and expressed in μmoles of P_{NA} per 1 ml. of solution. Simultaneously, the radioactivity of samples was estimated and expressed in counts per minute per 1 ml. of solution.

and the specific activity of nucleic acids was calculated (counts/min./μmole P_{NA}). The results are presented in Fig. 1.

In preliminary experiments toluene, xylene and thymol were used to inhibit any bacterial growth which might have interfered with the estimations. However,

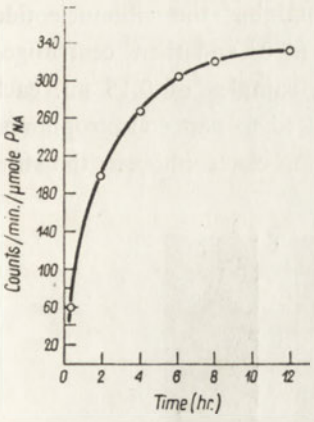

Fig. 1

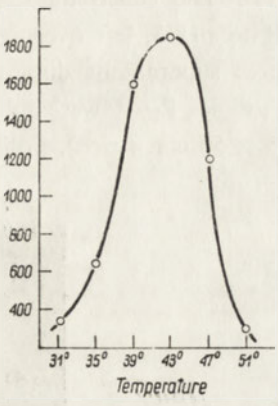

Fig 2.

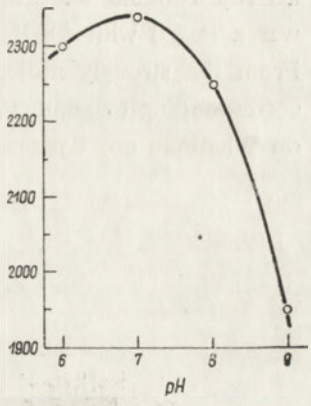

Fig. 3

Fig. 1. Effect of incubation time on the specific activity of nucleic acid. Each sample contained 2 g. of thymus homogenate, 2 ml. of 0.01 M-tris buffer, pH 7.3 and [^{32}P]orthophosphate (4×10^6 counts/min.). Temp. 37°.

Fig. 2. Effect of temperature on the specific activity. Each sample contained 1 g. of thymus homogenate, 2 ml. of 0.01 M-tris buffer, pH 7, and [^{32}P]orthophosphate (6×10^6 counts/min.). Incubation 12 hr.

Fig. 3. Effect of pH on the specific activity. Each sample contained 1 g. of thymus homogenate, 2 ml. of 0.01 M-tris buffer and [^{32}P]orthophosphate (1×10^7 counts/min.). Incubation 12 hr. at 39°

they were found to have no effect on the determinations and therefore they were not subsequently employed.

Effect of temperature. The procedure was identical as in the previous experiment except that only 1 g. of homogenate was used in order to increase the specific activity of the isolated nucleic acids. Basing on the results presented in Fig. 2, in further experiments the samples were incubated at 40°.

Effect of pH. This was studied at pH from 6 to 9 using tris buffer (Fig. 3).

Hydrolysis of the isolated RNA and specific activities of the individual ribonucleotides

A sample containing 4 g. of thymus homogenate, 8 ml. of 0.01 M-tris buffer, pH 7, and [^{32}P]orthophosphate (2×10^7 counts/min.) was incubated for 12 hr. at 39°. Then the nucleic acid was isolated according to Davidson & Smellie [5], dissolved finally in 0.01 M-tris buffer and dialysed for 7 days against water at 4° with constant stirring. The dialysed solution was then lyophilized and afterwards hydrolysed in 1 ml. of 1 N-KOH for 18 hr. at 37°. Before hydrolysis a sample was submitted to paper chromatography in Krebs & Hems [9] solvent system, and

then autoradiographed (Fig. 4). One radioactive RNA spot was found, on the start line.

From the hydrolysate DNA and proteins were precipitated by the addition of 60% $HClO_4$ to pH 1. The precipitate dissolved in 4 N-KOH showed no radioactivity (double background). The supernatant containing the ribonucleotides was adjusted with 4 N-KOH to pH 3, left overnight at 0° and then centrifuged. From the strongly radioactive supernatant duplicate samples of 0.15 ml. each, corresponding to about 0.7 μmoles P_{NA}, were submitted to paper electrophoresis on Whatman no. 1 paper (5 × 50 cm. strips). Following electrophoresis the strips

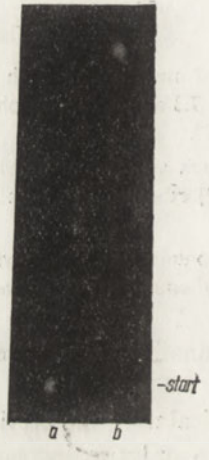

Fig. 4

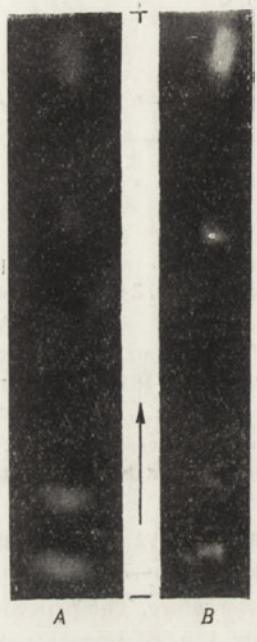

Fig. 5

Fig. 4. Autoradiogram of paper chromatogram of (*a*), non-hydrolysed RNA; (*b*), [^{32}P]orthophosphate
Fig. 5. Paper electrophoresis of RNA hydrolysate. (*A*), Autoradiogram; (*B*), photograph in UV

were left for 4 days in autoradiography. The developed autoradiograms showed a good separation of 4 spots of individual ribonucleotides (Fig. 5A). The distribution of these spots agrees with the distribution of spots localized in UV light (Fig. 5B). The spots were then cut out and eluted twice with 2 ml. of water at 100° for 15 min. After centrifugation the eluates were adjusted with water to the volumes of 4 ml. and the absorption spectra from 240 to 290 mμ were measured. An eluate of a piece of Whatman no. 1 paper saturated previously with acetate buffer and dried, served as a blank.

Table 1

Specific activities of individual ribonucleotides eluted from paper after electrophoretic separation

Compound	E_{260}	$\varepsilon_{260} \times 10^{-3}$	μmoles P_{RNA}/4ml.	Counts/min./4 ml.	Specific activity
AMP	1.008	15.0	0.27	855	3170
CMP	0.588	7.6	0.31	985	3180
GMP	1.020	11.8	0.34	1115	3280
UMP	0.615	9.9	0.25	810	3240

From the extinction values found at 260 mμ and the known [2] molar extinctions the concentration of individual ribonucleotides in the eluate was calculated and expressed in μmoles P_{RNA}. The determined radioactivity of the eluates was used to calculate the specific activities of the nucleotides (Table 1).

Incorporation of [^{32}P]orthophosphate into RNA isolated from thymus

Enzymic fraction. Thymus homogenate was ground with glass powder in a cooled mortar with 0.01 M-tris buffer, pH 7, added. After centrifugation at 32 000 *g* (Servall type RC Superspeed Refrigerated Centrifuge) the supernatant was diluted with 0.01 M-tris buffer to protein concentration of about 10 mg./ml., centrifuged again and left in ice for further experiments.

Ribonucleic acid. The spun down fraction of homogenate after separation of the enzymic fraction was transferred to a homogenizer, treated with 0.14 M-NaCl, stirred gently for 3 min. and centrifuged. After threefold repetition of this procedure, the combined extracts were centrifuged again and treated with 1 vol. of ethanol. Since large amounts of protein precipitated together with RNA, the precipitate was extracted twice with 10% NaCl solution for 15 min. at 100°. The centrifuged extracts were combined, treated with double volume of ethanol and left overnight at 0°. Precipitation and dissolving were repeated several times, the precipitated RNA being finally dissolved in 7.5 ml. of 0.01 M-tris buffer, pH 7. The RNA concentration was determined spectrophotometrically and corresponded to about 4.5 μmole P_{NA}/ml. The prepared RNA was examined chromatographically in Krebs & Hems solvent. After 18 hr. run only one spot on the starting line was found in UV light.

Two samples were prepared for the experiment. The first one contained RNA solution in the amount corresponding to 9 μmole P_{NA}, [^{32}P]orthophosphate (6×10^6 counts/min.), and 0.2 ml. of the enzymic fraction (2 mg. of protein). The second sample (control) consisted of the same amounts of RNA and [^{32}P]orthophosphate but with no enzyme added. Both samples were incubated for 12 hr. at 39° and then treated with 40% TCA. The precipitates were washed with 10%

TCA and neutralized with 1 N-NH_4OH. After extraction of RNA by means of 10% NaCl solution and repeated precipitation by ethanol, the specific activities of the samples were as follows: Whole sample 480 counts/μmole P_{NA}/min.; without the enzymic fraction, 180 counts/μmole P_{NA}/min.

DISCUSSION

The optimum conditions for the enzymic incorporation of [^{32}P]orthophosphate into nucleic acids were found to be: temp. about 43°, pH about 7. Below 31° and above 51° the activity of the enzyme was very low. [^{32}P]Orthophosphate was incorporated into RNA but not into DNA. This is in agreement with the observations of other workers [8, 1] that [^{32}P]orthophosphate is being incorporated into DNA only during mitosis. Autoradiography of the electrophoretically separated hydrolysis products of the isolated RNA showed that all four ribonucleotides were labelled to the same extent. The enzymic system was found to be present in the supernatant after centrifugation at 32 000 *g*.

SUMMARY

An enzyme system catalysing the incorporation of [^{32}P]orthophosphate into thymus RNA is present in the supernatant after centrifugation of thymus homogenate at 32 000 *g*. The optimum temperature is 43°, optimum pH 7.

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ENZYMY FOSFORYLUJĄCE KWASY NUKLEINOWE

I. WŁĄCZANIE [^{32}P]ORTOFOSFORANU W KWASY NUKLEINOWE GRASICY

Streszczenie

W supernatancie homogenatu grasicy po wirowaniu przy 32 000 *g* stwierdzono obecność enzymu katalizującego włączanie [^{32}P]ortofosforanu do kwasu rybonukleinowego. Optymalna temperatura reakcji wynosiła 43°, optymalne pH 7.

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THE ACTIVITY OF CYSTEINE DESULPHHYDRASE AND RHODANASE IN ANIMAL TISSUES

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One of the metabolic pathways of cysteine in the animal body is its degradation to hydrogen sulphide, ammonia and pyruvic acid (equation 1) catalysed by cysteine desulphhydrase [L-cysteine hydrogensulphide-lyase (deaminating)], described by Fromageot *et al.* [6] and Smythe [11]. Recently this enzyme has been the object of detailed studies [5, 7, 3] which indicated that the system catalysing the desulphurization of cysteine is more complex than it was previously supposed, and that the reaction products included also alanine and serine. The fate of the hydrogen sulphide formed has not been elucidated up to now, although certain data indicate that thiosulphate may be formed during oxidation of sulphide in animal tissues [2, 13]. Other authors [15] consider thiosulphate to be the main intermediate in the formation of sulphates. Recently, Szczepkowski [14] has suggested that rhodanase (thiosulphate:cyanide sulphurtransferase) can participate in the transformation of hydrogen sulphide to thiosulphate. This author found that rhodanase catalyses the formation of thiosulphate from sulphide and sulphite in the presence of cystine or oxidized glutathione (equation 2).

$$\text{L-Cysteine} \xrightarrow[\text{(phosphopyridoxal)}]{\text{desulphhydrase}} H_2S + NH_3 + \text{pyruvate} \qquad (1)$$

$$\text{Cystine} + H_2S + SO_3^{2-} \xrightarrow{\text{rhodanase}} 2 \text{ cysteine} + S\text{-}SO_3^{2-} \qquad (2)$$

Until recently rhodanase was thought to catalyse only the formation of thiocyanate from thiosulphate and cyanide [8, 12] and from disulphide and cyanide [13]. The enzyme was found in considerable amounts in animal tissues, which does not agree with the limited formation of thiocyanate. Reinwein [10] reported that no relation between the activity of the enzyme and the content of thiocyanate in the tissues could be detected. The suggestion of Szczepkowski explains the role of rhodanase in the metabolism of sulphide. If his view is correct, the tissues in which toxic hydro-

gen sulphide is formed should also contain an enzyme ensuring its rapid elimination, and high rhodanase activity should be present.

The purpose of this study was to compare the activity of cysteine desulphhydrase and rhodanase in various tissues of several species of animals and to determine whether their distribution follows a definite pattern. Moreover, the experimental findings might help to define the role of various tissues in the formation of hydrogen sulphide.

METHODS

Enzyme preparations

The experiments were performed on tissues isolated from adult animals of both sexes of the following species: hooded rats, guinea pigs, domesticated pigeons, and frogs (*Rana temporaria*). The animals were killed by decapitation and samples of the tissues were ground immediately in mortars with quartz sand and Sörensen phosphate buffer, pH 7.4 (10 ml. buffer per 1 g. tissue). After centrifuging the homogenate for 2 min. at 2000 r.p.m., the supernatant was used for determinations of the enzymic activity. In each case the dry wt. of 0.5 ml. of this extract was established to permit the calculation of activity per 1 g. of dry wt.

Determination of cysteine desulphhydrase activity

The method developed was based on the experiments of Smythe [11] and consisted in incubating the tissue extract with a solution of cysteine in Warburg flasks in an atmosphere of nitrogen; then the hydrogen sulphide formed was estimated.

To the main compartment of the Warburg flask 2 ml. of enzyme extract was added, and to the centre well 0.4 ml. of 5% solution of zinc acetate to trap the liberated H_2S. One side arm contained 0.5 ml. of 0.1 M-solution of L-cysteine (Fluka) and the other 0.5 ml. of 50% solution of H_3PO_4. The flasks were connected with the manometers, immersed in the water bath at 38° and flushed with nitrogen, 500 ml. of the gas being used for each flask. Then the solution of cysteine was tipped from the side arm and the mixture was incubated for 2 hr. with moderate shaking. Then the solution of H_3PO_4 from the other side arm was added to stop the reaction and release H_2S remaining in the incubation solution. In the controls, the cysteine solution was tipped together with H_3PO_4 when the incubation was terminated. To ensure complete absorption of H_2S in the centre wells, the flasks were left in the water bath for another 20 min. with vigorous shaking. The contents of the centre wells were transferred to a 50 ml. flask containing 30 ml. of 0.2% solution of zinc acetate, and then 5 ml. of 100 mg.% solution of *p*-amino-*NN*-dimethylaniline in hydrochloric acid, and 1 ml. of 2.7% solution of $FeCl_3$ were added. Under these conditions H_2S with *p*-amino-*NN*-dimethylaniline formed methylene blue [1]. In spite of thorough washing of the centre well, some of the ZnS precipitate always remained. Therefore to the well 5 drops of *p*-amino-*NN*-dimethylaniline and 1 drop

of $FeCl_3$ solutions were added, and the coloured solution which formed was combined with the main solution. The volume was adjusted with distilled water to 50 ml., and extinction at 670 mμ measured in a Coleman spectrophotometer using as control a reagent blank. The H_2S content of the sample was calculated from a standard curve. In cases in which cysteine desulphhydrase activity was low, the colour reaction was carried out directly in the centre well; then the solution was transferred to a test tube, adjusted to 5 ml., and the extinction was measured. The activity was expressed in μmoles of H_2S formed during 2 hr. incubation per 1 g. of dry wt. of tissue extract.

Determination of cysteine desulphhydrase activity in tissues with a very low content of the enzyme presented a special problem. During prolonged incubation small amounts of hydrogen sulphide may also be formed as the result of non-enzymic reactions which are not easy to eliminate in spite of control tests. Therefore, when the amount of H_2S formed after 2 hr. incubation with cysteine was smaller than 0.01 μmoles per sample, the absence of enzyme activity was recorded.

Determination of cysteine desulphhydrase activity by measuring the hydrogen sulphide produced is encumbered with an error due to the metabolism of this compound in tissue homogenates. However, it was found (Koj & Frendo, in preparation) that sulphide metabolism under anaerobic conditions and without the addition of cystine or sulphite is very low. Evaluation of cysteine desulphhydrase activity from the amount of ammonia or pyruvate formed is less reliable as alanine and serine [3] were also reported to be present in the reaction products.

Determination of rhodanase activity

This was estimated by the method of Sörbo [12]. To 1 ml. of 0.125 M-sodium thiosulphate, 0.5 ml. of 0.2 M-KH_2PO_4, 0.5 ml. of 0.25 M-KCN, and 0.5 ml. of tissue extract were added. After 5 min. incubation at 20° the reaction was stopped by adding 0.5 ml. of 40% solution of formaldehyde, followed by 2.5 ml. of 10% ferri nitrate solution. The precipitate was filtered off and extinction at 460 mμ was measured; to the blank sample formalin was added before incubation. When rhodanase activity was very high, the samples were 5-fold diluted with $Fe(NO_3)_3$ before the measurement. Hilger Uvispec spectrophotometer was employed and the amount of thiocyanate calculated from a standard curve.

Rhodanase activity was expressed in μmoles of thiocyanate formed during 5 min. incubation per 1 g. of dry wt. of tissue extract. Since accurate determinations of activity at very low levels were not possible, activities below 0.1 μmole CNS^{1-} per sample were recorded as "traces".

RESULTS AND DISCUSSION

The results of experiments are presented in Tables 1 and 2, the activity of enzyme being expressed in μmoles of the formed reaction products and in percentages of activity found in liver, as in all animals tested the liver possessed the highest activity.

The distribution of cysteine desulphhydrase activity in the body of animals was found to be rather irregular. Besides the liver, the kidney and skeletal muscle exhibit high activity, followed by stomach, brain and testis. The lowest activity of the enzyme was found in lung and spleen, and no activity in the erythrocytes of all animals studied. Considering the ratios of the weight of various organs to the total body weight it can be concluded that skeletal muscles are an important source of sulphide liberated from cysteine. In contrast to the results of Fromageot *et al.* [6] the presented experiments point to a fairly general distribution of cysteine desulphhydrase in the animal body; there were, however, distinct differences and, curiously enough, greater difference was found between rat and guinea pig, which are related, than between rat and pigeon or frog (Table 3).

Rhodanase activity was also found in the tissues of all studied animals. The highest activity was found in liver and kidney, followed by brain, testis, stomach and muscle. Relatively little rhodanase activity was found in intestine, spleen and lung, and in erythrocytes only traces of activity, if any. Of the animals studied, pigeon tissues had the lowest rhodanase activity.

On comparing the cysteine desulphhydrase and rhodanase activity a certain relationship between the distribution of the two enzymes can be seen. Generally, the tissues with high cysteine desulphhydrase activity possess also high rhodanase activity, and the absence of desulphhydrase is accompanied by low rhodanase

Table 1

Cysteine desulphhydrase and rhodanase activity in rat tissues

Mean values from 10 determinations ±S.D. are given, except for testis (6 determinations). The enzymic activity is also calculated in relation to the activity of the liver.

Tissue	Desulphhydrase		Rhodanase	
	(μmoles H_2S/g. dry wt. of homogenate/2 hr.)	Relative activity (%)	(μmoles CNS^{1-}/g. dry wt. of homogenate /5 min.)	Relative activity (%)
Liver	30.76 ± 6.21	100.0	882 ± 110	100.0
Kidney	9.52 ± 2.12	30.9	831 ± 56	94.2
Skeletal muscle	3.91 ± 0.16	12.7	84 ± 10	9.5
Heart muscle	2.09 ± 0.12	6.8	134 ± 10	15.5
Stomach	1.07 ± 0.25	3.4	186 ± 20	21.1
Intestine	0.46 ± 0.14	1.5	20 ± 4	2.2
Spleen	0.47 ± 0.13	1.5	27 ± 5	3.0
Lung	0.28 ± 0.08	0.9	66 ± 10	7.4
Brain	1.73 ± 0.32	5.5	209 ± 12	25.5
Testis	0.58 ± 0.06	1.9	384 ± 44	43.3
Erythrocytes	0	0	trace	less than 2

Table 2

Cysteine desulphhydrase and rhodanase activity in the tissues of guinea pig, pigeon and frog

The average values from 3 determinations and limit values are given. The enzymic activity is also calculated in relation to the activity of the liver

Animal and tissue	Desulphhydrase		Rhodanase	
	(μmoles H_2S/g. dry wt. of homogenate /2hr.)	Relative activity (%)	(μmoles CNS^{1-}/g. dry wt. of homogenate/5 min.)	Relative activity (%)
Guinea pig				
Liver	5.28 (4.46 — 6.65)	100.0	915 (735 — 1181)	100.0
Kidney	1.33 (1.17 — 1.43)	25.1	817 (729 — 920)	89.2
Skeletal muscle	1.07 (0.98 — 1.14)	18.3	34 (30 — 38)	3.7
Heart muscle	0.64 (0.56 — 0.78)	12.1	19 (14 — 25)	2.0
Stomach	0.23 (0.21 — 0.26)	4.3	189 (166 — 210)	20.6
Small intestine	trace	less than 2	trace	less than 2
Spleen	trace	less than 2	56 (49 — 60)	6.1
Lung	trace	less than 2	178 (136 — 220)	18.3
Brain	0.24 (0.20 — 0.28)	4.5	55 (45 — 68)	6.0
Testis	0.55 (0.33 — 0.73)	10.2	75 (64 — 89)	8.2
Erythrocytes	0	0	trace	less than 2
Pigeon				
Liver	10.82 (9.07 — 12.38)	100.0	81 (61 — 108)	100.0
Kidney	13.41(12.06 — 15.03)	123.0	110 (90 — 127)	135.8
Skeletal muscle	7.90 (6.15 — 9.40)	73.0	49 (35 — 61)	60.5
Heart muscle	7.09 (5.21 — 9.30)	65.5	61 (51 — 69)	75.3
Stomach mucosa	0.84 (0.73 — 0.95)	7.5	trace	less than 2
Small intestine	2.03 (1.84 — 2.49)	18.7	trace	less than 2
Spleen	0	0	trace	less than 2
Lung	0.33 (0.14 — 0.48)	3.1	trace	less than 2
Brain	1.32 (0.89 — 1.63)	11.4	14 (11 — 18)	17.3
Testis	3.13 (2.62 — 3.40)	28.9	31 (23 — 44)	38.3
Erythrocytes	0	0	0	0
Frog				
Liver	17.08(13.19 — 22.22)	100.0	1074 (882 — 1366)	100.0
Kidney	8.82 (8.12 — 9.31)	51.6	647 (541 — 781)	60.2
Skeletal muscle	4.07 (2.42 — 5.62)	23.2	230 (168 — 304)	21.4
Heart muscle	trace	less than 2	42 (30 — 54)	3.9
Stomach and intestine	3.21 (1.94 — 4.09)	18.7	279 (223 — 366)	25.9
Ovary	0.71 (0.50 — 1.04)	4.2	31 (23 — 35)	2.8
Erythrocytes	0	0	trace	less than 2

activity. This observation seems to indicate the participation of both enzymes in sulphur metabolism. A similar parallel distribution of both enzymes was also found in human tissues (Frendo, Koj & Górniak, in press). Some similarity of cysteine desulphhydrase and rhodanase activity in developing chick embryos was also observed (Borysiewicz, Frendo & Koj, in press), which seems to confirm the existence of a functional relation between the two enzymes.

Table 3

Cysteine desulphhydrase and rhodanase activity in liver and kidney of various animals

The enzymic activity is calculated in relation to the activity of rat liver or kidney, respectively.

Tissue	Activity of	Rat	Guinea pig	Pigeon	Frog
Liver	Cysteine desulphhydrase	100.0	17.1	35.1	55.5
	Rhodanase	100.0	103.7	9.1	121.7
Kidney	Cysteine desulphhydrase	100.0	13.9	140.8	92.6
	Rhodanase	100.0	98.3	13.2	76.6

However, it should be noted that not in all animal tissues such a definite relation between cysteine desulphhydrase and rhodanase activity is present. Exceptions include the testis in rat (Table 1), lung in guinea pig, and intestines in pigeon (Table 2). Moreover, the data in Table 3 show that high desulphhydrase activity not always is accompanied by proportionally high rhodanase activity (e.g. in pigeon). This discrepancy may be explained by the complex character of cysteine metabolism and also of its degradation products. It is known that hydrogen sulphide may be formed also by transamination of cysteine [4] and then metabolized by various enzymes, not only rhodanase [2, 13, 9]. In spite of this, the presented experiments demonstrate a general correlation between the contents of cysteine desulphhydrase and rhodanase in animal tissues, and support the postulates of Szczepkowski concerning the important role of rhodanase in the metabolism of hydrogen sulphide.

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SUMMARY

Cysteine desulphhydrase and rhodanase activity were determined in various tissues of rat, guinea pig, pigeon and frog. The data indicate a certain parallelism between the distribution of the two enzymes in the animal body and suggest a functional relationship between them.

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AKTYWNOŚĆ DESULFHYDRAZY CYSTEINY I RODANAZY W TKANKACH ZWIERZĘCYCH

Streszczenie

Oznaczono aktywność desulfhydrazy cysteiny i rodanazy w różnych tkankach szczura, świnki morskiej, gołębia i żaby. Uzyskane wyniki wskazują na pewną równoległość w rozmieszczeniu obu enzymów w organizmie zwierzęcym oraz przemawiają za funkcjonalnym związkiem obu enzymów.

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DECARBOXYLATION OF TYROSINE AND DOPA IN THE TISSUES OF *CELERIO EUPHORBIAE*

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Our previous studies [6, 1] indicated that in *Celerio euphorbiae* the transamination of tyrosine and 3,4-dihydroxyphenylalanine (DOPA) is insignificant. Neither *p*-hydroxyphenylpyruvate oxidase nor homogentisic acid could be detected. Thus it appears that in insects the tyrosine metabolism follows a different metabolic pathway than in mammals. The melanogenetic pathway characteristic for insects can proceed only in conditions of unlimited oxygen supply, and it is not compatible with the steady-state concept. On the other hand the large variations in tyrosine content in *C. euphorbiae* [2] indicate the existence of some important metabolic pathway of tyrosine other than melanogenesis. This route might involve the decarboxylation of tyrosine or DOPA followed by the oxidation of the respective amines.

The present paper deals with the occurrence of tyrosine and DOPA decarboxylases in the tissues of *C. euphorbiae*; for comparison, analogous experiments were performed with hog kidney, rabbit brain and adrenal, and carp brain.

MATERIALS AND METHODS

Materials. Feeding caterpillars, caterpillars at the beginning of pupation and pupae of *C. euphorbiae* diapauzing at 4° were homogenized with 3 vol. of 0.01 M-phosphate buffer, pH 7.0, for chromatographic analysis, or with 3 vol. of 0.24 M-thiourea in 0.01 M-phosphate buffer, pH 7.0, for manometric assays. The homogenates were centrifuged at 13 000 r.p.m. for 20 min. at 0° and the precipitate was discarded. The supernatant after removal of the lipid layer from the surface was used for experiments.

In the same way were prepared the homogenates of hog kidney, rabbit brain and adrenal, and carp brain.

Reagents. L-Tyrosine (Zakłady Farmaceutyczne, Warszawa), L-3,4-dihydroxyphenylalanine (Biuro Odczynników Chemicznych, Gliwice), pyridoxal phosphate

(Fluka, Switzerland), thiourea, sulphanilic acid (Fabryka Odczynników Chemicznych, Gliwice), ninhydrin (B.D.H.), dioxane (Xenon, Łódź), diazonium salt of sulphanilic acid prepared from sulphanilic acid.

Tyrosine and DOPA decarboxylases. Their activity was determined by the manometric technique and by paper chromatography. In the manometric method 2.3 ml. of tissue extract in thiourea - phosphate buffer was placed in the main compartment of the Warburg flask, and 0.7 μmole of pyridoxal phosphate (0.2 ml.) and 11 μmoles of tyrosine or DOPA (0.5 ml.), in the side arm. After equilibration of temperature the substrate was tipped from the side arm and the mixture was incubated in the atmosphere of air at 37°. The readings were taken at 5-min. intervals; after 30 min. 0.2 ml. of 6 N-H_2SO_4 was introduced through the side arm and after 15 min. the reading was taken. In the control samples the substrates were omitted.

For chromatographic analysis the incubation mixture contained: 2.3 ml. of the tissue extract in phosphate buffer, 0.7 μmole of pyridoxal phosphate (0.2 ml.) 11 μmoles of tyrosine or DOPA (0.5 ml.) and 0.2 ml. of 0.01 M-phosphate buffer, pH 7.0. The mixture was incubated for 30 min. at 37° under nitrogen, then deproteinized by boiling and kept for 10 min. on a boiling water bath. After cooling, the protein was centrifuged off; 0.2 - 0.4 ml. of the supernatant was applied on Whatman no 4. paper and developed in butan-1-ol - acetic acid - water (4:1:5, by vol.). The spots were located with 0.2% ninhydrin solution in butanol or with diazonium reagent [3] prepared by dissolving 50 mg. of sodium salt of diazosulphanilic acid in 15 ml. of cooled water - dioxane mixture (2:1, v/v). After spraying with diazonium reagent, the chromatograms were submitted to an atmosphere of concentrated ammonia; red spots indicated the presence of tyrosine, DOPA and their amines.

Determination of protein. The protein content was determined by the Mejbaum-Katzenellenbogen tannin method [5].

RESULTS AND DISCUSSION

With the manometric technique no activity of tyrosine decarboxylase was observed in the tissues of *C. euphorbiae* at the three development stages (Table 1). Likewise, the decarboxylation of tyrosine was not found in hog kidney, rabbit brain and adrenal, and carp brain. By the chromatographic method, no decarboxylation products were detected in the respective incubation mixtures.

High tyrosinase activity in insects suggested that the first stage of metabolic breakdown of tyrosine may consist in its oxidation to DOPA followed by decarboxylation. It was found that decarboxylation of DOPA takes place in *C. euphorbiae* at the feeding caterpillar stage. Under the conditions used the decarboxylation reaction was finished already after 15 min. of incubation, and the activity calculated in relation to protein was the same as in hog kidney. No activity, however, was observed in *C. euphorbiae* either at the beginning of pupation or in pupae. Also in rabbit brain DOPA decarboxylase could not be detected. These findings form

an addition to the comparative studies of Holtz & Westermann [4, 8] who proved the presence of this enzyme in the adrenals of ram, horse, beef, hog, guinea pig and rat, and also in *Tenebrio molitor*.

Table 1

The activity of tyrosine and DOPA decarboxylases

The manometric technique was used; for conditions see text. The amount of protein in incubated samples: feeding caterpillar, 9 - 13 mg.; caterpillar at the beginning of pupation, 28 mg.; pupa, 24 mg.; hog kidney, 25 mg. Incubation: 30 min. Average values from 4 experiments are given. In parentheses the limit values.

Tissue	L-Tyrosine decarboxylase	L-DOPA decarboxylase
	(µl. CO_2/100 mg. protein)	
C. euphorbiae		
feeding caterpillar	0	222.5 (190 — 244)
at beginning of pupation	0	0
pupae	0	0
Hog kidney	0	276 (254 — 300)
Rabbit brain	0	0

The presence of DOPA decarboxylase in feeding caterpillars was confirmed chromatographically; on spraying either with ninhydrin or with diazonium reagent, besides a trace of DOPA (R_F 0.23) a distinct spot of DOPA-amine (R_F 0.42) was revealed.

According to Murphy & Sourkes [7] D-DOPA, *m*-tyrosine, α-methyl-5-hydroxytryptophan, and α-methyl-2,5-DOPA inhibit the decarboxylation of L-DOPA in rat. To ascertain whether the lack of DOPA decarboxylase in caterpillars at the beginning of pupation and in pupae is not due to the presence of such inhibitors, experiments with dialysed extracts were performed. When respective enzymic preparations were previously dialysed for 48 hr. against 4 l. of 0.24 M-thiourea in 0.01 M-phosphate buffer at 4°, also no decarboxylation of DOPA was observed.

The presented results seem to exclude tyramine as an intermediate of tyrosine and the problem of the main metabolic pathway of tyrosine in *C. euphorbiae* remains unsolved. The demonstration of active DOPA decarboxylase exclusively in feeding caterpillars presents a new problem for further investigations.

SUMMARY

1. Tyrosine decarboxylase activity was not found in *C. euphorbiae* tissues.

2. DOPA decarboxylase activity was found only in feeding caterpillars; no activity was observed at the beginning of pupation and in pupae.

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DEKARBOKSYLACJA TYROZYNY I 3,4-DWUHYDROKSYFENYLOALANINY W TKANKACH *C. EUPHORBIAE*

Streszczenie

1. U gąsienicy, wrzeciona i poczwarki nie stwierdzono dekarboksylacji tyrozyny.
2. U gąsienicy aktywność dekarboksylazy 3,4-dwuhydroxyfenyloalaniny była taka sama jak w nerce wieprzowej, natomiast nie stwierdzono jej aktywności u wrzeciona i poczwarki.

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STUDIES ON THE ORNITHINE CYCLE IN THE TISSUES OF *HELIX POMATIA* DURING HIBERNATION

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In excreta of *Helix pomatia* Needham [3, 14] and Baldwin [1, 2] found, besides uric acid, variable amounts of urea. Therefore Baldwin postulated that in summer, under conditions of good water supply, the snail produces and excretes urea, while in winter to avoid osmotic disturbances it forms the insoluble uric acid. Baldwin has also suggested [2] that uric acid might be synthesized, as postulated by Wiener in 1902 [18], from two molecules of urea and a C_3-precursor. This mechanism was in those days almost generally accepted, and with respect to insects it was held until recently. The evidence for the synthesis of purine ring from urea in insects was provided by the work of Leifert [11] on excretion of nitrogen in *Antheraea pernyi*.

In the last 20 years the conception of uric acid synthesis has been changed fundamentally. Several studies, particularly those of Buchanan (for review see [5]) on bird tissues proved that the synthesis of purine involves C_1-precursor, glycine, and amino groups of glutamine and aspartic acid. The experiments of Desai & Kilby [7] suggested the same metabolic pathway in insects, and Heller & Jeżewska [9] demonstrated the formation of uric acid in Tussur moth (*Antheraea pernyi*) according to Buchanan's scheme. The results of Leifert have not been confirmed.

Therefore it was of interest to examine Baldwin's assumption of seasonal formation of uric acid from urea in snail. If this assumption is to be accepted, the formation of urea must first be proved. Baldwin found a very active arginase in snail tissues, and accepted as valid a correlation such as found in invertebrates, between the distribution of this enzyme and the ureotelic type of excretion. Cohen & Brown [6], however, demonstrated that the presence of one, or even of some enzymes of the ornithine cycle cannot be accepted as a satisfactory proof for ureotelism. The only reliable evidence is provided by the presence in the tissue of all the enzymes of the urea cycle leading from ornithine, citrulline, and arginine to urea. The present study was undertaken to look for this evidence in *Helix pomatia* during hibernation.

MATERIALS AND METHODS

The snails (*Helix pomatia*) were collected in autumn, maintained in the laboratory, and after incapsulation kept at the temperature of 5°. For the experiments, the snail shell was cut away and hepatopancreas, kidney and foot muscles were isolated, and after homogenization acetone-dried powders were prepared. The acetone-dried tissues were extracted in all-glass Potter homogenizer using 100 vol. of 0.1% cetyl trimethyl ammonium bromide (CTB) which facilitated the extraction of the studied enzymes [4]. The extracts were centrifuged at 5000 r.p.m. for 7 min. at 4° and the supernatants used for the enzymic assays.

Protein was determined by the Mejbaum-Katzenellenbogen tannin method [13]. Citrulline was assayed by the Fearon method as modified by Łazariew [12], and urea by the Fosse xanthydrol method as modified by Engel & Engel [8]. The arginine content was calculated from the amount of urea formed upon the addition of arginase which was obtained from beef liver after Ratner [15]. The arginase preparation contained 7 - 9 mg. of protein per 1 ml. and was diluted as required.

Formation of citrulline was also checked by paper chromatography in water-saturated phenol in the atmosphere of 0.3% ammonia; the spots were located with ninhydrin - acetone solution. For detecting urea, the chromatograms were developed in propane-1-ol - water (7:3, v/v) and sprayed with *p*-aminobenzaldehyde.

Reagents. Citrulline hydrochloride and arginine hydrochloride (L. Light); ornithine hydrochloride and L-aspartic acid (Riedel); ATP (Schwartz); cetyl trimethyl ammonium bromide (B.D.H.). Amino acid solutions were adjusted before use to appropriate pH. Carbamyl phosphate in the form of dilithium salt of 65 - 75% purity was prepared from potassium cyanate and potassium dihydrogen phosphate after Jones, Spector & Lipmann [10].

Formation of citrulline

The presence of ornithine carbamoyltransferase (carbamoylphosphate : L-ornithine carbamoyltransferase) in snail tissues was examined in the system containing in 2 ml.: 50 μmoles of tris buffer, pH 8.5; 20 μmoles of carbamyl phosphate (CAP); 20 μmoles of ornithine and 0.5 ml. of acetone-dried tissue extract (0.7 mg. of protein). The incubation was carried out for 30 min. at 30°, and was stopped by adding 2 ml. of 1 N-perchloric acid. After centrifugation citrulline was estimated in the supernatant.

The extracts from hepatopancreas catalysed the synthesis of citrulline from ornithine and CAP (Table 1); this was proved by citrulline estimation and confirmed chromatographically. The extracts used did not contain citrulline. Incubation mixtures without the addition of CAP did not synthesize citrulline, whereas in the systems without ornithine, without extract, or with boiled enzyme solution, some citrulline was formed indicating a non-enzymic synthesis. The attention to

Table 1

Ornithine carbamoyltransferase activity in the tissues of Helix pomatia *during hibernation*

Incubation mixture contained in 2 ml.: 20 μmoles of ornithine, 20 μmoles of carbamyl phosphate, 0.5 ml. of the extract from acetone-dried hepatopancreas (0.7 mg. of protein), kidney (0.75 mg. of protein) or muscle (0.78 mg. of protein), and 50 μmoles of tris, pH 8.5. Incubation: 30 min. at 37°. The figures are average values from 6 experiments with hepatopancreas and from 5 experiments with kidney and muscle. Figures in parentheses indicate limit values.

Sample	Citrulline formed	
	μmoles/sample	μmoles/mg. protein
Hepatopancreas		
Whole system	0.32 (0.29—0.34)	0.46
Boiled extract	0.04 (0.039—0.041)	0.06
Carbamyl phosphate omitted	0.0	0.0
Ornithine omitted	0.027 (0.02—0.031)	0.04
Extract omitted	0.03 (0.028—0.031)	0.04
Kidney		
Whole system	0.25 (0.2—0.32)	0.33
Muscle		
Whole system	0.15 (0.15—0.15)	0.19

non-enzymic synthesis was drawn by Reuter [17] and also by Reifer & Kleczkowski [16] who studied ornithine carbamoyltransferase in plants. The amount of citrulline formed was dependent upon the concentration of the enzyme; the maximum synthesis was observed after 20 min. of incubation (Fig. 1). The activity of ornithine carbamoyltransferase was the same at pH 8.5 and pH 7.2. The enzyme was found, at variance with the finding in vertebrates, not only in snail liver but in kidney and in foot muscle as well.

Formation of arginine

The incubation mixture contained in 1.2 ml.: 50 μmoles of tris buffer, pH 7.2; 5 μmoles of citrulline, 5 μmoles of ATP, 5 μmoles of aspartic acid, 5 μmoles of $MgSO_4$, 0.4 ml. of acetone-dried tissue extract (0.56 mg. of protein) and 0.2 ml. of arginase solution (0.53 mg. of protein). The mixture was incubated for 60 min. at 37° and the reaction was stopped by adding 1.5 ml. of 93% acetic acid. In the supernatant urea was determined by the xanthydrol method.

The extracts used contained traces of arginine but no synthesis of arginine from citrulline was observed either in hepatopancreas or in muscle tissues. The same results were obtained with extracts from fresh tissues, thus excluding the possibility of inactivation of the enzymic system during preparation of acetone-dried powder. To eliminate the eventual occurrence in snail tissues of some compound inhibiting arginine synthesis, analogous experiments were performed with the

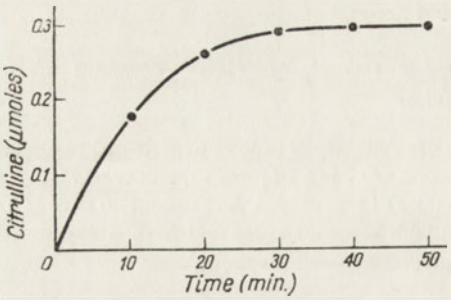

Fig. 1

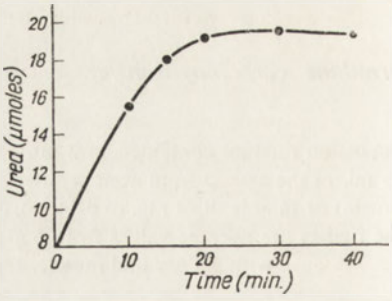

Fig. 2

Fig. 1. Synthesis of citrulline in the extracts from acetone-dried hepatopancreas. Conditions as described in Table 1

Fig. 2. Synthesis of urea in the extracts from acetone-dried hepatopancreas. Conditions as described in Table 2

enzyme from beef liver. This extract catalysed the synthesis of arginine from citrulline and aspartic acid, and the addition of the extract from snail tissues did not inhibit the synthesis.

Formation of urea

The activity of arginase was examined in the system containing in 1 ml.: 50 μmoles of tris buffer, pH 9.5, 20 μmoles of arginine, and 0.3 ml. of the enzymic extract (0.42 mg. of protein). The mixture was incubated for 30 min. at 37° and the reaction was stopped by boiling followed by addition of 1.5 ml. of 83% acetic acid. In the supernatant urea was determined by the xanthydrol method.

Table 2

Arginase activity in the tissues of Helix pomatia *during hibernation*

Incubation mixture contained in 1 ml.: 20 μmoles of arginine, 0.3 ml. of the extract from acetone-dried hepatopancreas (0.42 mg. of protein), kidney (0.45 mg. of protein) or muscle (0.18 mg. of protein), and 50 μmoles tris, pH 9.5. Incubation: 30 min. at 37°. The figures are average values from 4 experiments; in parentheses the limit values.

Sample	Urea formed	
	μmoles/sample	μmoles/mg. protein
Hepatopancreas		
Whole system	18.5 (18.2—19.2)	44
Arginine omitted	0.0	0.0
Extract omitted	0.0	0.0
Boiled extract	0.0	0.0
Kidney		
Whole system	10.5 (10—11)	23.3
Muscle		
Whole system	15.0 (14—16)	83.0

The extracts from all the tissues of *Helix pomatia* contained a very active arginase (Table 2), and arginine added was almost completely decomposed within 30 min. In the absence of either arginine or the enzyme extract, urea was not formed. In the amounts of extracts used for incubation no endogenic urea was detected. The rate of urea synthesis was proportional to the concentration of the enzyme; the greatest yield was observed after 15 - 20 min. (Fig. 2). The formation of urea and ornithine was confirmed chromatographically.

DISCUSSION

Ornithine carbamoyltransferase and very active arginase were found in snail tissues during hibernation, but the presence of an enzyme system catalysing the formation of arginine from citrulline and aspartic acid could not be demonstrated. No increase of arginine content was observed in the systems synthesizing citrulline from carbamyl phosphate and ornithine, even when ATP and aspartic acid were added. Despite high activity of arginase, no urea in snail nephridium was detected.

The presented results do not confirm Baldwin's assumption that the bulk of uric acid accumulating in snail during hibernation may be derived from urea.

SUMMARY

The enzymes of the ornithine cycle were studied in *Helix pomatia* during hibernation. Ornithine carbamoyltransferase and highly active arginase were found. The presence of an enzymic system synthesizing arginine from citrulline and aspartic acid could not be found.

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BADANIA NAD CYKLEM ORNITYNOWYM W TKANKACH ŚLIMAKA WINNICZKA W OKRESIE ZIMOWYM

Streszczenie

Badano obecność enzymów cyklu ornitynowego u ślimaka winniczka w okresie snu zimowego. Stwierdzono obecność transkarbamylazy ornitynowej oraz arginazę o wysokiej aktywności. Nie wykazano układu enzymatycznego, syntetyzującego argininę z cytruliny i kwasu asparaginowego.

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STUDIES ON SERUM GLYCOPROTEINS IN THE BLOOD OF VARIOUS SPECIES OF MAMMALS

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Few comparative studies on the amounts, composition and electrophoretic mobility of blood serum glycoproteins of various mammals were carried out. No data are available apart from the work of Sohar *et al.* [5] who studied the glycoproteins of dogs and rabbits by paper electrophoresis, and the work of Weimer & Winzler [8] on sugar composition of serum orosomucoid of several species of mammals.

The results presented in this paper indicate that glycoproteins assayed by chemical methods and by paper electrophoresis in the serum of mammals seem to be species-dependent.

MATERIALS AND METHODS

Blood samples were obtained by puncture of the jugular vein or of the left cardiac ventricle, from animals anaesthetized with ether, tranquilin or morphine, or stunned in the slaughter-house with electric current. In order to check the influence of anaesthetic agents, the blood of rabbits was taken from the marginal ear vein before and after anaesthesia; no differences in the electrophoretic patterns or in the sugar content of the studied proteins were found.

Paper electrophoresis. This was carried out at room temperature employing an apparatus described by Wieland & Fisher [9], and Whatman no. 1 paper strips 3.5 cm. wide. The buffer used was veronal-medinal solution, pH 8.6, I 0.05, potential 70 V, and time of electrophoresis 18 hr. For protein assays 5 - 7 μl. of serum was applied and for glycoproteins 20 - 30 μl. The amount of serum applied had no effect on the mobility of proteins.

When animal sera were examined for proteins and for glycoproteins, strips with human serum were placed for comparison in the same apparatus. After electrophoresis was completed, the electrophorograms were dried at 70° for 30 min. Then proteins were stained with Amido Black 10B, and glycoproteins by the method

Table 1

The distribution and electrophoretic mobility of blood serum glycoprotein fractions of various mammals

The average values of 3-6 determinations from 3 animals are given, except for lion (2 determinations from 1 animal). *M*, electrophoretic mobility; %, percentage of total serum glycoproteins.

	Mammal	Albumin		Alb - α_1		α_1		α_2		β_1		β		β_2		γ	
		M	%	*M*	%	*M*	%	*M*	%	*M*	%	*M*	%	*M*	%	*M*	%
1	Man	5.9	8.3	0	0	5.0	20.7	4.1	37.1	0	0	3.0	25.5	0	0	1.4	8.4
2	Monkey (*Rhesus macacus*)	6.0	9.4	0	0	4.6	26.2	3.5	36.5	0	0	2.3	14.2	0	0	1.6	13.7
3	White rat	0	0	5.3	53.2	0	0	3.4	14.1	0	0	2.4	24.4	0	0	1.6	8.3
4	White mouse	0	0	5.8	57.9	0	0	4.5	15.9	0	0	2.9	17.4	0	0	1.5	8.8
5	Guinea pig	4.6	21.5	0	0	4.3	26.0	3.8	24.7	0	0	2.5	14.6	0	0	1.2	13.2
6	Rabbit	6.5	13.6	0	0	4.9	28.7	4.3	25.9	0	0	3.7	16.2	0	0	1.5	15.6
7	European hare	6.4	9.2	0	0	4.7	30.8	3.6	30.2	0	0	2.8	17.8	0	0	1.6	12.0
8	Dog	6.5	5.9	0	0	5.1	13.0	4.3	41.3	2.9	13.3	0	0	2.3	15.1	1.2	11.4
9	Silver fox	6.3	10.2	0	0	5.4	17.8	4.4	22.6	3.5	14.9	0	0	3.0	25.9	1.3	8.6
10	Cat	6.6	9.0	0	0	4.8	18.8	4.2	36.7	0	0	3.2	20.2	0	0	1.4	15.3
11	Lion	6.7	18.5	0	0	5.3	9.0	4.4	46.8	0	0	3.2	16.2	0	0	1.6	9.5
12	Brown bear	6.1	16.4	0	0	5.3	24.0	3.9	30.9	0	0	2.8	20.7	0	0	1.6	8.0
13	Domestic cattle (1-2 year old)	6.1	9.5	0	0	4.8	43.2	4.2	13.8	0	0	2.9	17.8	0	0	1.6	15.7
	Domestic cattle (under 1 year)	6.2	11.7	0	0	4.8	48.3	4.2	13.7	0	0	3.0	17.9	0	0	1.7	8.4
14	Sheep	6.1	13.6	0	0	4.9	29.9	3.8	25.3	0	0	2.7	15.6	0	0	1.9	15.6
15	Pig	5.9	9.1	0	0	0	0	3.8	58.3	0	0	2.2	16.0	0	0	1.5	16.6
16	Horse	6.0	15.9	0	0	4.1	40.2	3.3	15.4	0	0	1.7	15.8	0	0	1.2	12.7

of Ceriotti [1] as modified by Radola [4]. Densitometric estimations of electrophorograms were made with a Photovolt model 525 apparatus using 545 filter.

The mobility of glycoprotein fractions was calculated by the method of De Wael & Punt [6] for determinations of the mobilities of haemoglobins.

Chemical analysis. Hexoses bound with total serum proteins or with mucoproteins only were assayed by the anthrone method of Dżułyńska & Piekarska [2]. Mucoproteins were isolated according to Graff *et al.* [3]. Proteins were determined by the biuret method [7] employing crystalline bovine serum as standard. Mucoproteins used for protein determinations were isolated from 4 ml. of serum.

RESULTS

The results of electrophoretic separation of glycoproteins are presented in Table 1. It was found that serum glycoprotein fractions of various animals differ markedly in their electrophoretic mobility, distribution, and even in their number.

The largest amounts of substances staining with Schiff's reagent were present in α_1- and α_2-glycoprotein fractions, or as in murine animals, in fractions with a mobility intermediate between that of albumins and α_1 called subsequently the Alb-α_1 fraction; smaller amounts were found in fractions corresponding to β--globulin and the smallest in the albumin and γ-glycoprotein fractions.

Certain fractions present in human serum were not found in some of the species of animals. For instance, in pigs only one α-fraction was present with mobility corresponding to that of the human α_2-fraction. In mice and rats, on the other hand, instead of the albumin and the α_1-fractions, large amounts of the Alb-α_1 fraction were found. In sera of other animals, e.g. dogs and foxes, additional fractions occur. In the region between the α_2- and γ-globulins two fractions were found, designated β_1- and β_2-glycoproteins.

These results seem to suggest that the mobilities and quantitative relations of the glycoprotein fractions are specific for animal species (Fig. 1). Family similarities were apparent only in some species, e.g. in mice and rats, and in dogs and foxes.

Further information on the animal serum glycoproteins was given by chemical assays of hexoses bound with total proteins and mucoproteins. As can be seen from the data presented in Table 2, the hexose content varies markedly. For instance, in rabbits the content of hexoses bound with total protein was 79.6 mg./100 ml., and in pigs it was 246.6 mg./100 ml. Analysis of variance (with a single classification according to species of animal) showed significant and very large differences in the content of hexoses bound with serum proteins, giving in Fisher's test F 138.2, strongly outstanding relative to random variability.

The percentages of hexoses in total protein also differed in various species of animals although these differences were less pronounced (F 32.6). Since a group

Table 2

Protein-bound hexoses in serum of various mammals

The mean values ± S.D. are given. In parentheses the under and upper limits of confidence interval.

	Mammal	Age	No. of animals	Hexoses in total protein		No. of animals	Hexoses in mucoproteins	
				mg./100 ml. serum	g./100 g. protein		mg./100 ml. serum	g./100 g. protein
1	2	3	4	5	6	7	8	9
1	Man	20 - 30 years	20	114.8 ± 9.2 (109.5 ; 119.8)	1.70 ± 0.07 (1.67 ; 1.73)	20	12.7 ± 0.72 (11.73 ; 13.37)	16.55 ± 1.12 (15.82 ; 17.14)
2	Monkey (*Rhesus macacus*)	unknown	13	165.6 ± 45.8 (141.3 ; 189.9)	1.95 ± 0.19 (1.85 ; 2.05)	13	24.62 ± 5.62 (21.08 ; 28.16)	16.50 ± 1.05 (15.84 ; 17.67)
3	White rat	3 - 6 months	43	124.1 ± 8.3 (121.5 ; 126.7)	1.83 ± 0.06 (1.81 ; 1.85)	11	9.29 ± 0.25 (9.11 ; 9.47)	—
4	White mouse	2 - 3 months	8 (pooled samples)	130.0 ± 1.4 (127.4 ; 132.6)	2.10 ± 0.07 (1.97 ; 2.23)	—	—	—
5	Guinea pig	4 - 6 months	11	102.7 ± 6.6 (98.0 ; 107.4)	1.70 ± 0.06 (1.66 ; 1.74)	—	—	—
6	Rabbit	5 - 6 months	21	79.6 ± 9.2 (75.6 ; 83.6)	1.64 ± 0.09 (1.60 ; 1.68)	10	6.89 ± 0.66 (6.49 ; 7.29)	11.64 ± 0.48 (11.35 ; 11.93)
7	European hare	unknown	16	93.2 ± 37.6 (78.3 ; 108.1)	1.87 ± 0.49 (1.58 ; 2.16)	15	12.28 ± 3.64 (10.10 ; 14.46)	13.44 ± 1.14 (12.75 ; 14.13)
8	Dog	unknown	14	109.6 ± 10.5 (103.3 ; 115.9)	1.59 ± 0.07 (1.55 ; 1.63)	8	10.0 ± 0.97 (9.12 ; 10.88)	12.56 ± 0.72 (11.73 ; 13.39)

1	2	3	4	5	6	7	8	9
9	Silver fox	1 year	19	89.0 ± 10.2 (83.9 ; 94.1)	1.62 ± 0.11 (1.57 ; 1.67)	15	5.47 ± 0.36 (5.26 ; 5.68)	12.93 ± 1.03 (12.34 ; 13.52)
10	Cat	unknown	8	137.4 ± 20.4 (119.2 ; 155.6)	2.49 ± 0.21 (2.36 ; 2.62)	8	11.45 ± 3.98 (7.88 ; 15.02)	14.95 ± 0.46 (14.54 ; 15.36)
11	Lion	2 years	1	133.0	2.15	1	9.1	12.50
12	Brown bear	1 - 2 years	6	98.0 ± 9.5 (87.1 ; 108.9)	1.52 ± 0.07 (1.44 ; 1.60)	6	9.87 ± 0.59 (9.20 ; 10.54)	15.12 ± 0.70 (14.14 ; 16.10)
		over 2 years	15	136.0 ± 5.8 (133.7 ; 138.3)	1.7 ± 0.06 (1.67 ; 1.73)	15	7.90 ± 0.18 (7.80 ; 8.0)	12.20 ± 0.29 (12.03 ; 12.37)
13	Domestic cattle	1 - 2 years	16	114.7 ± 9.2 (109.6 ; 119.8)	1.64 ± 0.04 (1.59 ; 1.67)	16	7.26 ± 0.18 (7.16 ; 7.36)	12.20 ± 0.11 (12.14 ; 12.26)
		under 1 year	12	78.6 ± 4.5 (72.3 ; 84.9)	1.56 ± 0.04 (1.49 ; 1.63)	5	5.26 ± 0.22 (4.95 ; 5.57)	11.98 ± 0.24 (11.65 ; 12.31)
14	Sheep	1 - 3 years	18	101.2 ± 7.4 (98.3 ; 104.1)	1.55 ± 0.07 (1.52 ; 1.58)	18	8.68 ± 0.55 (8.40 ; 8.96)	12.83 ± 0.30 (12.68 ; 12.98)
15	Pig	1 - 1.5 years	26	246.6 ± 24.6 (238.1 ; 255.1)	3.34 ± 0.11 (3.30 ; 3.38)	26	24.15 ± 3.68 (22.63 ; 25.67)	17.43 ± 0.62 (17.17 ; 17.89)
16	Horse	3 - 10 years	13	124.4 ± 8.94 (120.8 ; 128.0)	1.65 ± 0.02 (1.64 ; 1.66)	13	7.15 ± 0.18 (7.04 ; 7.26)	12.03 ± 0.37 (11.80 ; 12.26)

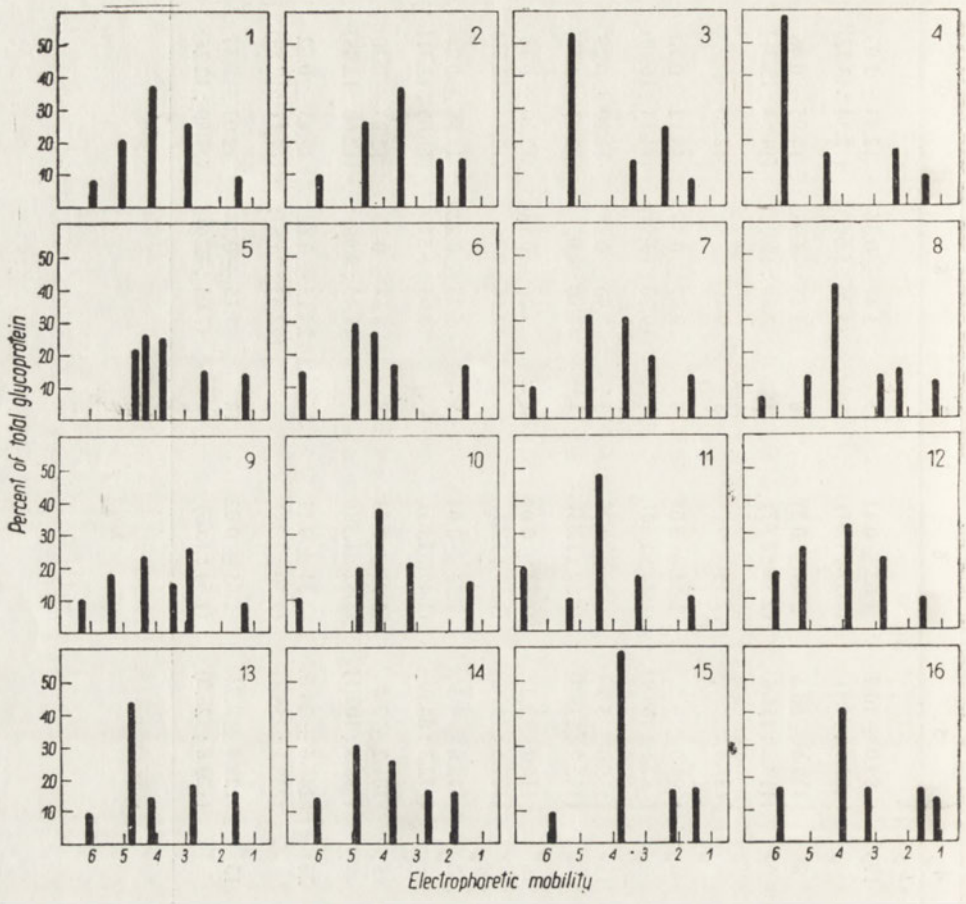

Fig. 1. The distribution and electrophoretic mobility of serum glycoprotein fractions. Details see Table 1. (1), Man; (2), monkey; (3), white rat; (4), white mouse; (5), guinea pig; (6), rabbit; (7) european hare; (8), dog; (9), silver fox; (10), cat; (11), lion; (12), brown bear; (13), domestic cattle; (14), sheep; (15), pig; (16), horse

of 6 animals with similar means was distinct, variance in it was analysed separately; difference was significant although not very conspicuous (F 10.5).

As in total protein, marked differences were found also in the content of hexoses in serum mucoproteins of different mammals and the analysis of variance gave F 98.9. There were no apparent separate groupings of species.

The percentual content of hexoses in mucoproteins also showed marked differences in the examined species (F 121.8). However, after discarding three divergent values the rest appeared to form a fairly compact group; nevertheless, analysis of variance showed significant differences (F 9.6). This indicates that the content of hexoses, although variable in a narrower range, is also a species characteristic.

DISCUSSION

The distribution of the glycoprotein fractions found in the sera of rabbits and dogs could not be compared with the data of Sohar *et al.* [5]. These authors, using different methods, did not separate the two β-glycoprotein fractions in dogs and the two α-fractions in rabbits.

The adopted nomenclature of animal glycoprotein fractions was tentatively based on the fractions occurring in human serum although the mobility of the fractions in animal sera differed very distinctly from that of the analogously named human fractions. Also the amounts of glycoprotein fractions in animal sera were not the same as in human sera. In some species, e.g. in rats, mice and pigs, there were only four fractions present, but in the canine species (dogs and foxes), there were six.

The small number of examined mammals does not permit conclusions as to similarities in the electrophoretic patterns and in percentages of hexoses bound with serum proteins, among animals belonging to the same family or order.

In the electrophoretic patterns of glycoproteins from the sera of cows of different age, no differences related to the age of the animals were found (Table 1, no. 13). On the other hand, marked differences were observed in the amount of hexoses bound with proteins (Table 2, no. 13). Therefore in this study mature but still young animals were selected, although sometimes, e.g. in cats, hares and monkeys, such a selection could not be achieved. The marked scatter of the results may therefore have been due to age differences. In hares prolonged stress due to fright and sudden change of conditions may have also played a part.

The differences in percentages of hexoses in serum mucoproteins of different species of animals may be an indication of differences in the chemical composition of mucoproteins. This was pointed out by Weimer & Winzler [8] who found distinct differences in the hexoses content of orosomucoid in six species of animals.

SUMMARY

1. Blood sera of 16 species of mammals were studied.

2. Paper electrophoresis demonstrated species-dependent differences in mobility, quantitative distribution and even in the number of glycoprotein fractions.

3. The amounts of hexose bound with protein and found in mucoprotein were also species-dependent.

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BADANIA RÓŻNICUJĄCE GLIKOPROTEIN SUROWICY KRWI RÓŻNYCH GATUNKÓW SSAKÓW

Streszczenie

Elektroforeza bibułowa surowic 16 gatunków ssaków wykazała znaczne różnice w ruchliwości, ilościowym rozkładzie, a nawet w ilości frakcji glikoproteinowych w zależności od gatunku zwierzęcia. Gatunkowe różnice stwierdzono również w ilości heksoz związanych z białkiem oraz z mukoproteidami.

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OCCURRENCE OF γ-L-GLUTAMYL-L-TYROSINE AND γ-L-GLUTAMYL-L-PHENYLALANINE IN SEEDS OF *LUPINUS ANGUSTIFOLIUS* AND *LUPINUS ALBUS*

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In a previous communication [15] the isolation by paper electrophoresis of two acidic peptides from seeds of *L. albus* has been reported. Both peptides, called K_1 and K_2, after acid hydrolysis gave in electrophoresis several ninhydrin-positive spots which might indicate either their complex amino acid structure or the presence of some contaminations accompanying the peptides through separation and hydrolysis. To characterize the structure of these peptides, they were isolated in larger amounts. As it appeared on the electrophorograms that the amount of the two peptides in *L. angustifolius* seeds was much higher than in *L. albus*, the former was used as the main source of peptides.

The isolation of the peptides was carried out from alcohol extract by three-step procedure: (1), removal of sugars and salts on resin Dowex 50 × 4; (2), separation of the peptides from amino acids on resin CG-45; (3), separation of peptide K_1 from K_2 on cellulose column.

From 5 kg. of *L. angustifolius* seeds 1.50 g. of crystalline peptide K_1 (m.p. 225 - 226°) and 0.15 g. of crystalline peptide K_2 (m.p. 170°) were obtained. The amino acid composition of these compounds appeared to be very simple. In hydrolysates of K_1, by electrophoretic and chromatographic methods only glutamic acid and tyrosine, and of K_2, glutamic acid and phenylalanine were found. From the hydrolysates the amino acids were isolated in pure form and identified as L-glutamic acid and L-tyrosine from K_1, and L-glutamic acid and L-phenylalanine from K_2. The identifications were carried out by chromatography, paper electrophoresis, determination of melting point and optical rotation, and finally by ultraviolet and infrared spectra (Fig. 1, 2, 3).

In both peptides, glutamic acid was *N*-terminal, while in K_1 tyrosine and in K_2 phenylalanine were the *C*-terminal amino acids. The solubility and electrophoretic properties of K_1 and K_2 suggested their dipeptide structure. Assuming that hydrolysis has only liberated but not altered the amino acids, the obtained results

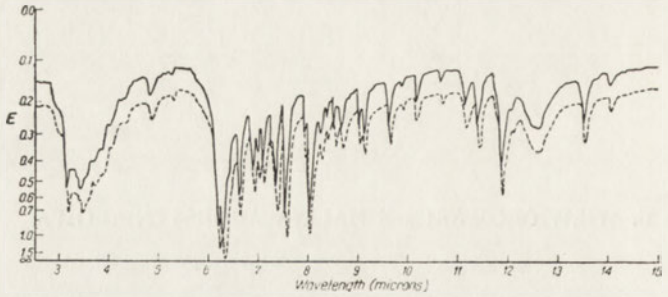

Fig. 1. IR-spectra of L-tyrosine (0.5 mg. in 300 mg. KBr); (- - -), isolated from peptide K_1; (—), standard sample

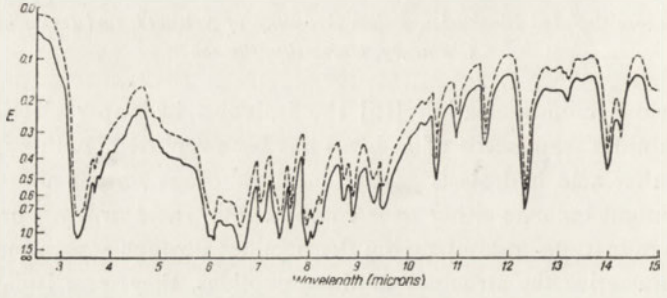

Fig. 2. IR-spectra of L-glutamic acid (1.5 mg. in 300 mg. KBr); (- - -), isolated from peptide K_1 and K_2; (—), standard sample

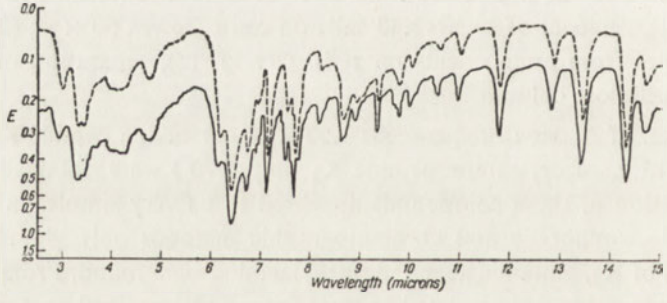

Fig. 3. IR-spectra of L-phenylalanine (1 mg. in 300 mg. KBr); (- - -), isolated from peptide K_2; (—), standard sample

indicate that peptide K_1 is glutamyltyrosine and peptide K_2 glutamylphenylalanine. Still, it was not clear whether α- or γ-carboxyl group of glutamic acid is engaged in peptide-bond formation.

According to Waley [14] the γ-linked peptides of glutamic acid have electrophoretic mobilities over twice as large as the α-linked ones. Peptides K_1 and K_2

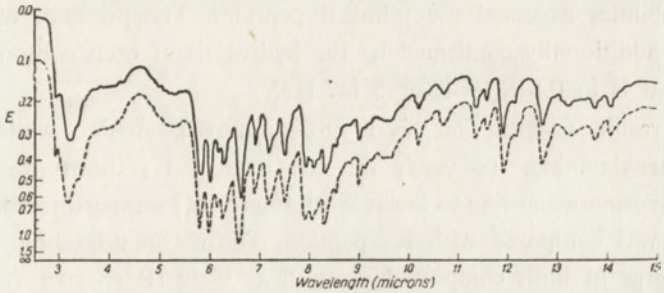

Fig. 4. IR-spectra of γ-L-glutamyl-L-tyrosine (1 mg. in 300 mg. KBr); (- - -), peptide K_1 isolated from *L. angustifolius* and *L. albus*; (—), chemically obtained [11] and sample received from S.G. Waley

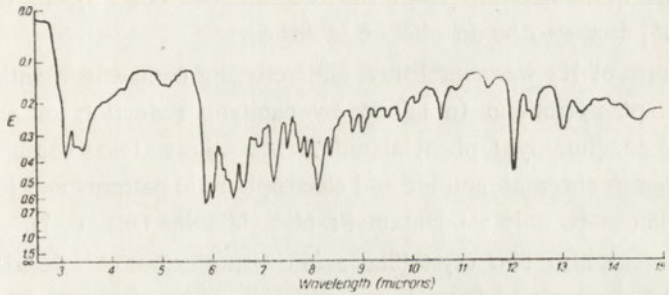

Fig. 5. IR-spectrum of α-L-glutamyl-L-tyrosine (0.8 mg. in 300 mg. KBr)

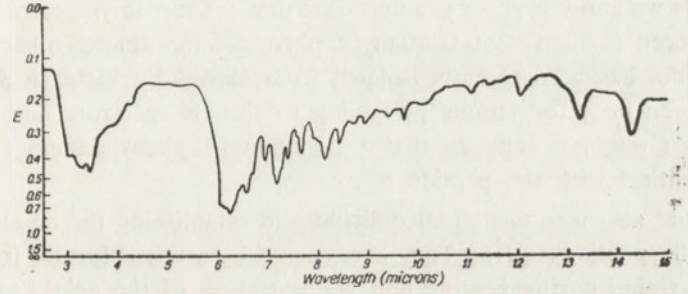

Fig. 6. IR-spectrum of γ-L-glutamyl-L-phenylalanine (peptide K_2) isolated from *L. angustifolius* (1 mg. in 200 mg. KBr)

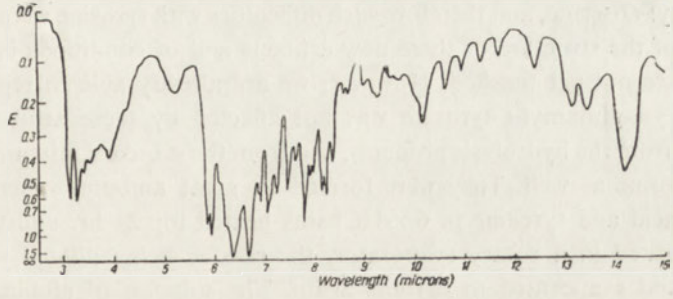

Fig. 7. IR-spectrum of α-L-glutamyl-L-phenylalanine (1 mg. in 300 mg. KBr)

have the mobilities expected for γ-linked peptides. The presence of γ-glutamyl linkages was additionally confirmed by the hydrolysis of each compound in mild conditions that is in 0.5 N-HCl for 5 hr. [13].

All these results suggest that K_1 is γ-L-glutamyl-L-tyrosine while K_2 is γ-L-glutamyl-L-phenylalanine. To verify the structure of K_1 the synthesis of γ-L--glutamyl-L-tyrosine according to Sorm & Rudinger [11] was performed. The synthetic dipeptide was compared with K_1 peptide. The chromatographic and electrophoretic patterns of both compounds as well as their IR spectra (Fig. 4) were found to be identical. A comparison was also made with α- and γ-L-glutamyl-L--tyrosine (a gift from Dr. S.G.Waley). The K_1 peptide was identical with the γ-compound but quite different from the α-compound (Fig. 5). In this way the structure of K_1 seems to be definitely established.

The structure of K_2 was established indirectly, by comparison with a synthetic α-L-glutamyl-L-phenylalanine (obtained by catalytic reduction of a commercial carbobenzoxy-α-L-glutamyl-L-phenylalanine). As these two compounds differ from each other in chromatographic and electrophoretic patterns and in IR spectra, the K_2 peptide can be only γ-L-glutamyl-L-phenylalanine (Fig. 6, 7).

From *L. albus* also two crystalline acidic peptides were isolated; they were identical with those isolated from *L. angustifolius*. Their yield was 20 mg. K_1 and 2.5 mg. K_2 from 200 g. of seeds.

As far as we know it is for the first time that γ-L-glutamyl-L-tyrosine of plant origin has been isolated. γ-L-Glutamyl-L-phenylalanine among other four γ-glutamyl peptides has been recently isolated from onions by Virtanen & Matikkala [12]. Although no informations pertaining to the IR spectrum and the melting point were given, we suppose that γ-L-glutamyl-L-phenylalanine from onions may be identical with the peptide K_2.

We should also mention initial difficulties in establishing the amino acid composition of the peptide K_1 [15]. They were caused by artifacts formed from glutamic acid and tyrosine during heating and concentration of the acidic solutions. We found, however, in the literature some informations proving the tendency of glutamic acid to form γ-esters [4, 3, 16, 5] but they concerned only γ-monoethyl ester and γ-glutamyl-*O*-serine, and therefore such difficulties with tyrosine were unexpected. The studies of the structure of these new artifacts and of conditions in which they are formed are not yet finished. However, we are already able to report that the structure of γ-L-glutamyl-L-tyrosine was not affected by these artifacts, as they were formed from the hydrolysis products, and from the standard mixture of glutamic acid and tyrosine as well. They were formed in great amounts when a mixture of glutamic acid and tyrosine in 6 N-HCl was heated for 24 hr. under reflux and then concentrated in a rotary evaporator, the residue being diluted several times with water and evaporated to dryness again. The solution of glutamic acid and tyrosine treated in this way and submitted to electrophoresis gave only traces of

glutamic acid beside a large band of neutral amino acids and a broad band of a basic fraction in the position corresponding to histidine (Fig. 8). In the neutral amino acids fraction beside tyrosine there was at least one artifact, as after hydrolysis in sealed tube and concentration in a vacuum desiccator over KOH, some glutamic acid was also present. The basic fraction on rechromatography separated into two distinct spots, which on hydrolysis gave tyrosine and glutamic acid. On the chromatogram of the hydrolysate also some other spots were present, suggesting further transformations of both amino acids during the applied procedure. Since we were not aware of the above facts when publishing our preliminary communication, there are some discrepancies between our previous and present publication.

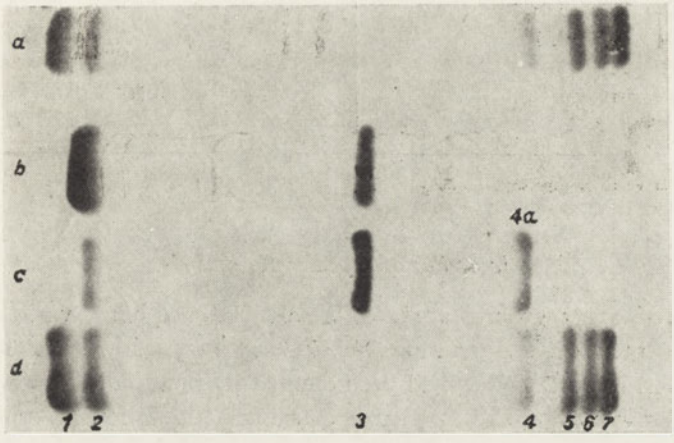

Fig. 8. Electrophorogram: (*a*) and (*d*), mixture of standard amino acids; (*b*), hydrolysis products of peptide K_1 (hydrolysis in sealed tube); (*c*), hydrolysis products of peptide K_1 (hydrolysis under reflux) *1*, Aspartic acid; *2*, glutamic acid; *3*, fraction of neutral amino acids; *4*, histidine; *4a*, basic artifact; *5*, arginine; *6*, lysine; *7*, ornithine. Conditions of electrophoresis: pyridine buffer [7], potential 3 KV, duration of separation 1.5 hr.

In addition, these differences are aggravated by contaminations which had been present in the samples of K_1 and K_2 obtained from electrophorograms, especially in the preparation of K_2 which had contained large amounts of the peptide K_1 and aspartic acid.

EXPERIMENTAL

The values of the melting points are given uncorrected. The IR spectra (Fig. 1 - 7) were made on a Perkin Elmer Infracord 137 spectrophotometer in potassium bromide. Approximately 1 mg. of the dried amino acid or peptide was ground with 300 mg. KBr and the mixture was submitted for 5 min. to a pressure of 12 000 kg. per cm.^{2}. Details of paper chromatography and paper electrophoresis are given in Table 1.

Table 1

The rate of movement of glutamyl dipeptides in circular paper chromatography and paper electrophoresis

The chromatograms were made on Whatman no. 3 paper; solvent systems: (*a*), butane-1-ol-acetic acid - water (4:1:5, by vol.), 8 hr.; (*b*), pyridine - acetic acid - ethyl acetate - water (5:1:5:3, by vol.). (*c*), Paper electrophoresis in pyridine buffer, pH 5.85 [7]. The rates of movement in relation to glutamic acid (R_{glu}) are given.

Compound	R_{glu} in solvent		
	a	*b*	*c*
Glutamic acid	1.00	1.00	1.00
K_1, (γ-L-glu-L-tyr)	1.35	1.29	0.57
K_2, (γ-L-glu-L-phe)	1.49	1.30	0.62
α-L-Glu-L-tyr	—	1.49	—
α-L-Glu-L-phe	—	1.50	—
Basic artifact	—	—	0.79*

* This value is expressed in R_{arg}

Isolation of peptides K_1 and K_2

Five kg. of finely ground seeds of *Lupinus angustifolius*, variety Obornicki, grown in the plant-breeding station I.U.N.G. in Przybędowo was defatted with ether for 36 hr. After the ether had been completely removed, the powder containing 12% water was submitted to ethanol extraction, the following procedure being used.

Three samples of about 1.7 kg. each were mixed with about 2 litres of 85% ethanol and ground in a porcelain mill for about 10 hr. The suspension was then transferred into glass vessels and allowed to stand until the residue settled down; then the supernatant was carefully decanted. To the residue fresh portions of 70% ethanol were added, and left standing, with occasional shaking, for 24 hr., and then the supernatant was decanted. In this way the extraction was continued for 3 weeks. The total amount of 70% ethanol used was 25 litres. Each portion of extract was passed through filter paper and concentrated under reduced pressure at 35°. The total volume of pooled concentrated extracts was 4.5 litres. To precipitate the eventual admixture of proteins the solution was heated to boiling and filtered, then extracted with ether for 24 hr. in a perforator. This procedure resulted in a decrease of the volume of the water layer to 3.2 litres. To remove the traces of ether the water solution was aerated under reduced pressure, using a water pump. pH of the extract was 6.5. For the isolation of peptides from the extract a three-step procedure was applied.

Removal of sugars and salts on resin Dowex 50 × 4. As the amount of sugars in the extract was very high it appeared impossible to use column chromatography. The clogging of the column was so strong that in spite of 0.5 atm. pressure and

heating to 60° used it was found impossible to elute the adsorbed material. For this reason a preliminary removal of sugars and salts was necessary. The procedure was as follows: 450 ml. of Dowex 50 × 4 resin (H^+ form) and 600 ml. of the extract were mixed for 24 hr. Then the supernatant, which contained no ninhydrin-positive compounds, was rejected. The resin was transferred to a column 100 × 2 cm. and washed with water until the washings turned neutral. The water eluate containing mainly sugars was rejected. Amino acids and peptides adsorbed on the resin were eluted with 1200 ml. of ammonia and the eluate concentrated under reduced pressure at 40° to about 250 ml.

The combined concentrated eluates (total vol. 1.2 litre) were submitted once more to column chromatography on Dowex 50 × 4 (H^+ form). A column (100 × 2 cm.) was filled with 350 ml. of resin suspension in water, the column being regenerated for each operation with 6 N-HCl according to Buchanan [2]. The partially purified extract (400 ml.) was poured into the column. After the resin had been imbibed with the extract, the column was washed with water until neutral. The elution of amino acids and peptides was by a gradient of ammonia solution. The increasing ammonia concentration was applied by running 1 lit. of 4 N-NH_4OH solution into 1 lit. of water. Two litres of eluent was used in each operation. All this procedure was carried out under pressure of 0.3 atm. and the flow-rate was 16 drops per minute. The effluent was collected in 10 ml. fractions, each fraction being examined for ninhydrin-positive compounds, and by high-voltage electrophoresis for peptides. From 3 columns, about 2 litres of effluent have been collected containing peptides, acidic and neutral amino acids and very small quantities of basic amino acids as well. The greater part of basic amino acids was separated and rejected. The eluate was then concentrated under reduced pressure to about 400 ml. and submitted to column chromatography on CG-45 resin.

The separation of peptides from amino acids. The CG-45 resin (Haas & Rohm Comp.) was prepared as follows: water suspension of 100 ml. of the resin was poured onto the column (65 × 1.2 cm.) and after the water has passed through, 5 vol. of 1 N-HCl was applied. After the excess of acid was removed with water the resin was washed with 0.5 N-NaOH until chloride-free, and then with water until neutral. The whole procedure was repeated 5 times, then the ionite was transformed into acetate form with 2 vol. of 4 N-acetic acid, the excess of acid being removed with water.

Fifty ml. of the concentrated eluate adjusted with 2 N-acetic acid to pH 5 was placed on the CG-45 resin column. Basic and neutral amino acids were eluted with 850 ml. of water, glutamic and aspartic acids with 800 ml. of 0.5 N-acetic acid, and the peptide fraction with 1200 ml. of 2 N-acetic acid. Successive fractions were checked by paper electrophoresis. The fractions containing the peptides were combined, concentrated under reduced pressure and dried in vacuum. From 400 ml. of the concentrated eluate from Dowex 50 × 4 column, 4.1 g. of crude peptides was obtained.

The separation of peptides on cellulose column. A column (130 × 4 cm.) was filled with 400 g. of cellulose powder and washed with a mixture of butane-1-ol - acetic acid - water (4:1:5, by vol.) for 24 hr. In 20 ml. of the same solution 2 g. of crude peptides was dissolved and applied on the column. The elution was performed with the same solvent system the flow-rate being 3 drops per minute. Fractions of 7 ml. were collected. Total amount of the eluates was 1500 ml., of which 550 ml. contained peptides. The results of the separation are presented in Table 2.

Table 2

The separation of peptides on cellulose column

Eluent solution: butane-1-ol - acetic acid - water (4:1:5, by vol.).

Fraction	Volume (ml.)	Compounds found
I 0 — 750 ml.	750	no ninhydrin-positive compounds
II 750 — 950 ml.	200	peptide K_2
III 950 — 1040 ml.	90	mixture of peptide K_1 and K_2
IV 1040 — 1240 ml.	200	peptide K_1
V 1240 — 1320 ml.	80	peptide K_1 and traces of amino acids
VI 1320 — 1500 ml.	180	no ninhydrin-positive compounds

From fractions no. II and IV which contained pure peptides the solvent was removed under reduced pressure and crystallization effected from water. After removal of the mother liquor the crystals were washed with anhydrous methanol. Fraction no. III which contained the mixture of peptides was submitted to repeated separations on a suitably smaller cellulose column. Fraction no. V which contained the peptide K_1 and traces of amino acids was also purified chromatographically.

Yields. From 5 kg. of *L. angustifolius* seeds 1.5 g. of peptide K_1 and 0.15 g. of peptide K_2 were obtained.

γ-L-*Glutamyl*-L-*tyrosine* (*peptide* K_1)

Hydrolysis. (*a*) For 1 mg. of peptide, 0.5 ml. of 5.6 N-HCl, temp. 100°, and 24 hr. in sealed tube were applied. The hydrolysate was evaporated to dryness in a vacuum desiccator over KOH and the residue dissolved in water. Some tyrosine then crystallized.

The hydrolysate from 30 mg. of peptide K_1 was separated on the resin CG-45 (acetate form; column 35 × 1.4 cm.). Tyrosine was eluted with water, and glutamic acid with 0.5 N-acetic acid. About 12 mg. crystalline tyrosine and about 8 mg. crystalline glutamic acid were obtained. Both amino acids were identified by paper chromatography, paper electrophoresis and finally by IR spectra (Fig. 1, 2).

The specific rotation $[\alpha]_D^{20}$ was determined in 1% solutions in hydrochloric acid, and it was found to be for tyrosine in 5 N-HCl -9.4° and for glutamic acid

in 6 N-HCl +30°. This proves that both amino acids of the peptide K_1 have the L-configuration.

(*b*) Per 1 mg. of peptide 0.2 ml. of 0.5 N-HCl, boiling water bath, 5 hr. Under these mild hydrolysis conditions the peptide K_1 underwent complete hydrolysis giving glutamic acid and tyrosine, as proved by chromatographic and electrophoretic analysis.

(*c*) Per 1 mg. of peptide 1.4 ml. of 5.6 N-HCl, temp. 120° (oil bath), 24 hr. under reflux. The hydrolysate was evaporated to dryness in a vacuum desiccator at 40°. To remove traces of HCl the residue was dissolved in water and again evaporated to dryness; this was repeated 4 times. On the electrophorogram the hydrolysis products gave 3 ninhydrin-positive spots of different intensity (Fig. 8). An artifact having electrophoretic mobility similar to that of histidine, was separated by paper chromatography (butane-1-ol - acetic acid - water, 4:1:5, by vol.) into two different substances which on hydrolysis in sealed tube with 5.6 N-HCl, 100°, 24 hr., gave tyrosine, glutamic acid, and a small amount of other substances.

A very similar electrophoretic pattern was obtained from a mixture of tyrosine and glutamic acid heated with 6 N-HCl and evaporated.

Determination of N-*terminal and* C-*terminal amino acids.* The dinitrophenyl (DNP) method of Sanger [10] for *N*-terminal, and hydrazinolysis [1, 8] for *C*-terminal amino acids were used. On the chromatogram of the hydrolysate of DNP-peptide K_1 only DNP-glutamic acid was present, and tyrosine was the only free amino acid obtained after hydrazinolysis.

The synthesis of γ-L-*glutamyl*-L-*tyrosine and comparison with the peptide* K_1. The dipeptide was prepared from commercial carbobenzoxy-γ-monohydrazide-L--glutamic acid (Mann, New York) and from commercial L-tyrosine (La Roche, Switzerland) by the method of Sorm & Rudinger [11]. It was found by chromatography that crude product contained small amounts of substances which seemed to be identical with the α-isomer. After purification on cellulose column the dipeptide melted at 225° whether alone or in a mixture with peptide K_1.

The synthetic dipeptide and peptide K_1 had identical chromatographic and electrophoretic patterns. Their identity was finally established by comparison of UV and IR spectra (Fig. 4). Our synthetic and natural dipeptides were also identical with γ-L-glutamyl-L-tyrosine received from Dr. S.G.Waley (Fig. 5) but they were different from α-L-glutamyl-L-tyrosine; one sample of this preparation was kindly sent by Dr. S.G.Waley, and the second one was obtained by catalytic reduction of carbobenzoxy-α-L-glutamyl-L-tyrosine (Mann, New York).

γ-L-*Glutamyl*-L-*phenylalanine* (*peptide* K_2)

The hydrolysis of the peptide K_2 was carried out by the method (*a*) and the products were separated on the CG-45 resin. From 30 mg. of peptide K_2 about 11 mg. of crystalline phenylalanine and about 8 mg. crystalline glutamic acid were obtained. These amino acids were identified by chromatography and electropho-

resis, and finally by IR spectra (Fig. 2 and 3). Optical rotation $[\alpha]_D^{20}$ of phenylalanine (1% aqueous solution) was -31° and of glutamic acid (1% 6 N-HCl solution) +31°. These data indicate that both amino acids have L-configuration.

The *N*- and *C*-terminal amino acids were estimated as above and it was found that glutamic acid was the *N*-terminal, and phenylalanine the *C*-terminal amino acid.

Comparison of the properties of peptide K_2 with synthetic α-L-glutamyl-L--phenylalanine was carried out using a preparation obtained from the commercial carbobenzoxy derivative (Mann, New York) by Pd-catalysed reduction. The crude product after twofold crystallization had the melting point at 180°. On chromatography this substance was found to be homogeneous but diffferent from peptide K_2. In pyridine phase [6] in which γ-glutamyl dipeptides are readily separated from the α-glutamyl ones, the synthetic α-L-glutamyl-L-phenylalanine and the peptide K_2 had different mobilities. The synthetic preparations of α-L-glutamyl--L-tyrosine and α-L-glutamyl-L-phenylalanine could not be separated from each other in this solvent but they showed higher mobility than the peptides K_1 and K_2 (Table 1). This proves the peptide K_2 to be γ-L-glutamyl-L-phenylalanine. The IR spectra of the synthetic α-L-glutamyl-L-phenylalanine also differed much from those of the peptide K_2 (Fig. 6, 7).

Peptides K_1 and K_2 from seeds of L. albus

In these experiments the seeds of a sweet variety of *L. albus* (from plant breeding station I.U.N.G. in Przybędowo) were used. The isolation and identification of peptides was carried out as for the seeds of *L. angustifolius*, and the same results were obtained.

The authors wish to express their gratitude to Dr. S.G. Waley for the generous gift of γ-L-glutamyl-L-tyrosine and α-L-glutamyl-L-tyrosine.

SUMMARY

From seeds of *L. angustifolius* and *L. albus* two acidic peptides K_1 and K_2 were isolated in crystalline form, the yield being 0.04% of dry wt. of seeds. The identification of hydrolysis products, *N*- and *C*-terminal amino acids, melting points and infrared spectra indicated the peptide K_1 to be γ-L-glutamyl-L-tyrosine and K_2, γ-L-glutamyl-L-phenylalanine. Their structure was finally proved by comparison with chemically obtained corresponding dipeptides. Some attention was paid to the artifacts which were formed during heating and evaporation of the acidic mixture of glutamic acid with tyrosine.

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WYSTĘPOWANIE γ-L-GLUTAMYLO-L-TYROZYNY I γ-L-GLUTAMYLO-L-FENYLOALANINY W NASIONACH *L. ANGUSTIFOLIUS* I *L. ALBUS*

Streszczenie

Z nasion *L. angustifolius* i *L. albus* wyizolowano w stanie krystalicznym dwa kwaśne peptydy (K_1 i K_2), stanowiące ok. 0.04% masy nasion.

Na podstawie analizy produktów hydrolizy, wyników oznaczeń *N*- i *C*-końcowych reszt aminokwasowych, temperatur topnienia oraz widma w podczerwieni ustalono, że peptyd K_1 jest γ-L-glutamylo-L-tyrozyną, a peptyd K_2 — γ-L-glutamylo-L-fenyloalaniną.

Zwrócono uwagę na artefakty tworzące się w trakcie podgrzewania i zagęszczania kwaśnych roztworów kwasu glutaminowego i tyrozyny.

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CHEMICAL DETERMINATION OF DNA IN ANIMAL TISSUES*

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Reliable methods for chemical determination of DNA are imperative for progress within many fields of research. The available methods are based on determination of one of the three components of DNA, i.e., phosphate, UV absorbing bases, or deoxyribose. Since the two former components are also present in RNA, the accuracy of the methods based on analysis of these substances is dependent on the possibility of separating RNA and DNA.

The method of Schmidt & Thannhauser [20] employs determination of the P content. Several reports may be found in the literature demonstrating that complete separation of RNA and DNA is not always achieved, and that other P-containing substances under certain circumstances may interfere with the analysis [4, 12, 6, 24]. Also for the Ogur & Rosen method [17] an interference of contaminants and incomplete separation of RNA and DNA has been reported [24, 19, 18, 11, 1, 23].

For many reasons the method originally developed by Schneider [21] seems to be the most attractive approach. In this method purine deoxyribose is determined colorimetrically with the Dische [7] diphenylamine reaction. Due to the high specificity of this reaction (cf. Burton [2]) separation of DNA and RNA is not necessary. The original Schneider method has been modified by using perchloric (PCA) instead of trichloroacetic acid (TCA) for nucleic acid extraction (Schneider, Hogeboom & Ross [22]). Some authors prefer the very sensitive Ceriotti indol reaction [3] for deoxyribose estimation.

When *deoxyribose* is determined in extracts of DNA or of nucleic acids from animal tissues, made according to the different methods mentioned above, the highest value is obtained with the Schneider, and the lowest with the Schmidt-Thannhauser extracts (de Deken-Grenson & de Deken [5]). This finding inevitably arouses suspicion with regard to the low yielding methods, although it is no proof of their invalidity, since they were not developed on the basis of deoxyribose deter-

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mination. De Deken-Grenson & de Deken also found that the yield varied with the extraction temperature. This finding was confirmed, and further extended in the author's laboratory. Thus, using dry powders of liver it was found that when extraction was performed at 70° the DNA content was higher in rabbit liver than in rat liver, whereas at 90° the opposite relation prevailed (Løvtrup & Roos [16]). This finding prompted a more detailed study of the processes occurring during the treatment of DNA with hot PCA.

Before an account of these results is given, it may be mentioned that various devices have been introduced to improve the results obtained with the methods discussed above. Likewise, other analytical principles have been employed (for DNA, e.g., microbiological assay, isotope dilution) but it is beyond the scope of the present paper to discuss these aspects.

Extraction of DNA with hot PCA

At the outset it should be mentioned that the Burton [2] modification of the Dische diphenylamine reaction has been employed in all the experiments reported here. In this method the acid extracts, together with the colorimetric reagent, are

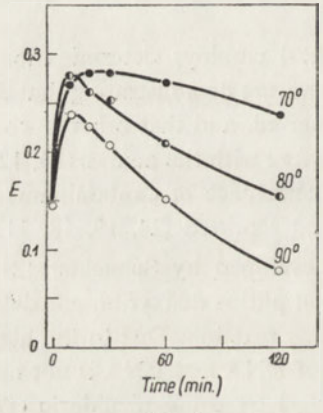

Fig. 1

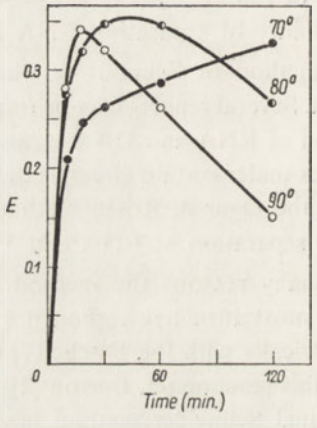

Fig. 2

Fig. 1. The effect of treatment with hot PCA on the determination of DNA with diphenylamine
Fig. 2. Determination of DNA with diphenylamine in PCA extracts of rat liver dry powder

incubated overnight at 30°. It is indeed possible that some of the values of the early points in our curves might have been different if the brief treatment at 100° of the original Dische method had been employed.

When high-polymeric DNA is treated at different temperatures with PCA, the results shown in Fig. 1[1] obtain. It is seen that a certain time of treatment with PCA is necessary to obtain maximum extinction. Depolymerization and hydrolysis of

[1] Figures 1 - 8 are reproduced from S. Løvtrup and K. Roos, *Biochim. Biophys. Acta*, 53, 1 - 10, 1961, by kind permission of Elsevier Publishing Company.

DNA presumably occur during this period, but the curves hardly reflect these processes, since a certain treatment with hot PCA is necessary in order to obtain maximum extinction even with low-molecular deoxyribose compounds (cf. below). After 10 min. a decrease in extinction is seen at 80° and 90°; at 70° the maximum occurs after 20 min. The rate of decrease is strongly dependent on the temperature;

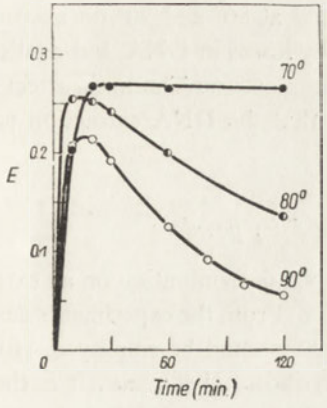

Fig. 3

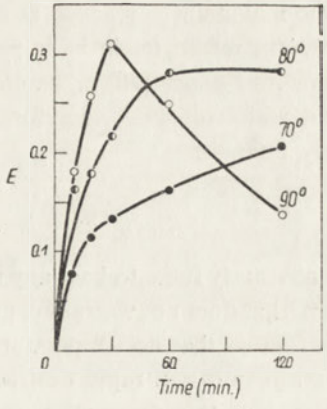

Fig. 4

Fig. 3. Determination of DNA with diphenylamine in PCA extracts of rabbit liver dry powder
Fig. 4. Determination of DNA with diphenylamine in PCA extracts of rat liver dry powder previously treated with cold PCA according to Ogur & Rosen [17]

at 90° it is so high as to make the maximum value considerably lower than those obtained at the lower temperatures. The decreasing extinction must represent some process in which deoxyribose is destroyed.

The results obtained when the same experiment is carried out with rat liver powder are shown in Fig. 2. Between the curves in Fig. 1 and 2 several differences are noted. Some of these demonstrate the influence of the extraction process, and some the differences of the nucleic acids reaction towards the treatment with hot PCA.

The extinction reaches a maximum at a later time than in Fig. 1, showing that at the beginning the rate of extraction exceeds the rate of destruction. These conditions prevail during 2 hr. at 70°, since no maximum is obtained. The lack of a maximum (cf. also Fig. 3) is remarkable and significant, because it indicates that little or no destruction occurs before the DNA has been extracted. As will be shown later, this phenomenon must be taken into account when DNA is determined. Comparison of the two Figures also shows that the standard DNA gives the lowest extinction at 90°, and liver powder the highest; the actual difference between the three temperatures in Fig. 2 will thus be further exaggerated when the DNA content is calculated by comparison with the standard.

When rabbit liver powder is used, the resulting curves are as shown in Fig. 3. It is seen that the rate of destruction is higher for this type of DNA, which also seems

to be easier to extract. These two factors together result in higher values at 70° than at 90°, in agreement with the observations mentioned above.

In order to get a notion of the influence of the cold PCA treatment of the Ogur-Rosen procedure on the subsequent DNA extraction with hot PCA, the above experiments were repeated with liver powders treated according to this method. Only the results with rat liver powder will be shown here (Fig. 4). It is seen that at 90° the maximum is reached at a later time, and at 80° and 70° no maximum is obtained. The results might be in part explained by losses in DNA, but as discussed in the previous paper [16], apparently no great loss occurs. The main effect of the treatment with cold PCA therefore seems to be that the DNA extraction is made extremely difficult.

Kinetic experiments on DNA and dry powders

It is obviously futile to base any method for DNA determination on an extraction procedure that does not warrant complete extraction. From the experiments described above it follows that no temperature lower than 90° should be employed. However, at this temperature, a rapid destruction of deoxyribose takes place. It is therefore imperative that this destruction should be somehow accounted for. Fortunately, this turned out to be rather easy since the decrease in extinction was found to follow a first order scheme:

$$\log \frac{E_t - E_\infty}{E_0 - E_\infty} = -k \times t.$$

In this equation k is the velocity constant, t the time, and E_0, E_t, and E_∞ the extinction at $t = 0$, $t = t$, and $t = \infty$, respectively.

For a solution of high-polymeric salmon sperm DNA the results shown in Fig. 5 were found. Using decadic logarithms, the following values were obtained: $E_0 = 0.442$; $E_\infty = 0.070$; and $k = 0.0059$ min.$^{-1}$. The DNA concentration, based on P analysis, was 37.7 μg. per ml., thus 11.7 extinction units per mg. DNA. The relation of this value to the theoretically expected extinction will be discussed later. It should be noted that due to a mistake an erroneous value was employed for the DNA content in the previous publication [16]. The value of 9.5 for the extinction per mg. DNA given in that paper is therefore wrong.

When similar kinetic experiments are performed on liver powders, the resulting curves are necessarily more complicated, because of the interference of the extraction process occurring simultaneously. It was found, however, that for rat liver the points obtained after more than one hour of extraction neatly followed a first order scheme (Fig. 6). After this period of time all reacting deoxyribose apparently is dissolved, and the only process to be accounted for is the destruction proper. The curve obtained using these points had the following characteristics: $E_0 = 0.543$; $E_\infty = 0.021$; and $k = 0.0040$ min.$^{-1}$. The lower rate constant was interpreted to mean that rat liver DNA is much more stable toward hot PCA

than is salmon sperm DNA. Results to be reported later in this paper show that this interpretation was incorrect.

The calculated value of E_0 cannot be considered to represent the true zero value, unless all DNA had been subjected to destruction according to the rate described by the curve. The experiments in the preceding section showed that unextracted DNA is not destroyed at this high rate. In order to introduce a correction it will be assumed that unextracted DNA is completely stable, so that our task is reduced to calculate the destruction of dissolved deoxyribose. For the first 10 min. period, the average amount of deoxyribose present is taken to be half of that indicated

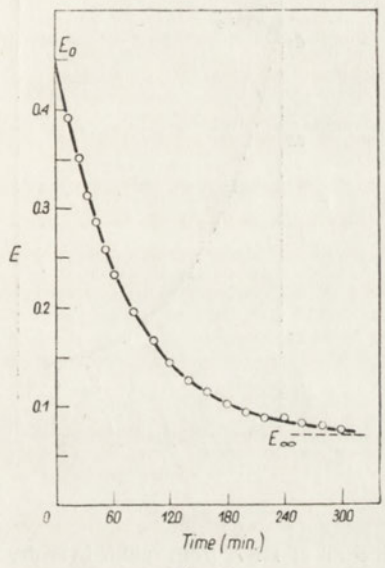

Fig. 5

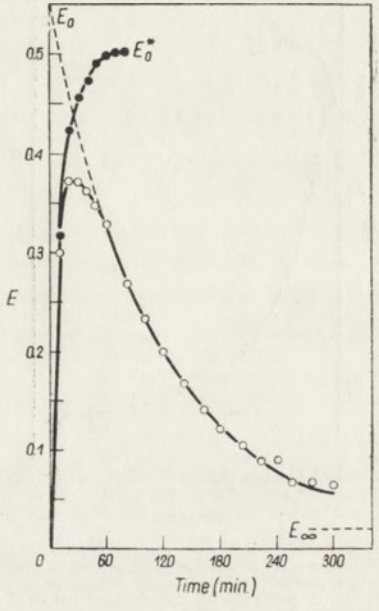

Fig. 6

Fig. 5. Kinetic analysis of the decrease of extinction (destruction) in the reaction of DNA with diphenylamine as a result of treatment with PCA at 90°. For significance of E_0 and E_∞, see text

Fig. 6. Kinetic analysis of the extraction and destruction of DNA from rat liver dry powder with PCA at 90°. For significance of E_0, E_0^* and E_∞, see text

by the extinction value found at the end of the interval. From this value and the first order equation it is possible to calculate the decrease in extinction, which has to be added to the observed value. For the next 10 min. period the average between the first and the second reading is taken to represent the deoxyribose content, a new decrease is calculated, and this value, together with the first decrease, is added to the 20 min. extinction value. Continuing this procedure, a curve approaching a constant value is obtained (Fig. 6). This constant value, $E_0^* = 0.502$, an approximation of the true E_0, is reached after extraction for one hour.

Very similar results were obtained with rabbit liver powder (Fig. 7). The following values were found: $E_0 = 0.477$; $E_\infty = 0.058$; and $k = 0.0079$ min.$^{-1}$.

The value of E_0 was calculated as described above except that for the first 10 min. period a value was used equal to 3/4 of the 10 min. extinction value. This has been done in all cases where the first point was a maximum value, as a smooth curve drawn through the point must lie considerably above the straight line connecting the origin and the first point. E_0^* was found to be 0.448.

Powders from other tissues were also extracted. Kidney DNA was found to be completely extracted in 20 min., as shown in Fig. 8. The velocity constant was found to be 0.0052 min.$^{-1}$ for both rat and rabbit kidney. Heart DNA was slightly

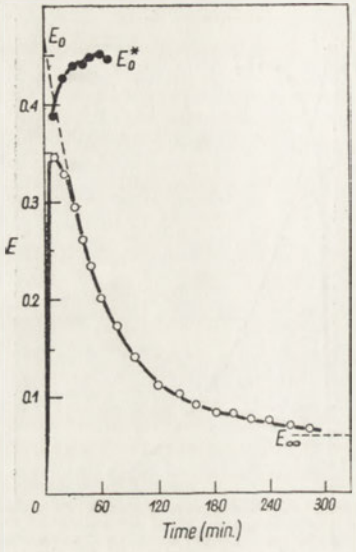

Fig. 7

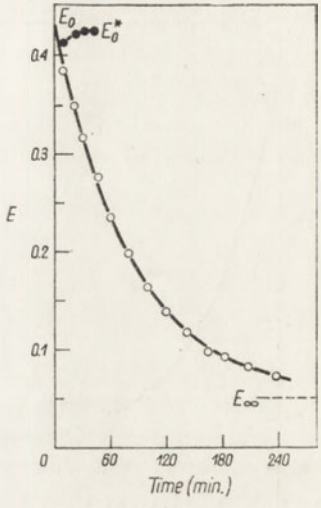

Fig. 8

Fig. 7. Kinetic analysis of the extraction and destruction of DNA from rabbit liver dry powder with PCA at 90°. For significance of E_0, E_0^* and E_∞, see text

Fig. 8. Kinetic analysis of the extraction and destruction of DNA from rat kidney dry powder with PCA at 90°. For significance of E_0, E_0^* and E_∞, see text

more difficult to extract, at least 30 min. being necessary. The value of k was found for both species to be 0.0065 min.$^{-1}$. Brain DNA requires 40 - 50 min. for complete extraction. Also for this organ k was identical for rat and rabbit: 0.0070 min.$^{-1}$. Calf thymus DNA was found to be as easy to extract as kidney DNA. The value of k was 0.0051 min.$^{-1}$

Kinetic experiments on low-molecular deoxyribose compounds

In order to get a better understanding of the mechanism of the process of deoxyribose destruction the kinetic experiments were repeated with various low-molecular substances. For deoxyribose proper the obtained results are shown in Fig. 9. It will be noticed that, maybe contrary to expectation, the points do not follow a first

order curve throughout. Treatment with hot PCA for some time seems to be necessary to obtain maximum extinction. The same phenomenon was observed with deoxyadenosine and deoxyguanosine. The following values obtain for the curve shown for deoxyribose: $E_0 = 0.630$; $E_\infty = 0.002$; $k = 0.0034$; 0.0745 μmole per ml. For deoxyadenosine and deoxyguanosine the constant was likewise found to be 0.0084. From the E_0 values the extinctions per μmole were calculated to be: 8.45; 8.52; and 8.21, respectively. It will be realized that there is no justification for introducing a corrected E_0 value for these substances, as they were dissolved from the outset. Curves for the two purine deoxyribosides are shown together with those for the pyrimidine deoxyribosides, deoxycytidine and thymidine, in Fig. 10. The latter substances are seen to give next to no extinction at zero time,

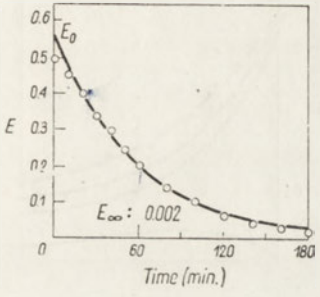

Fig. 9

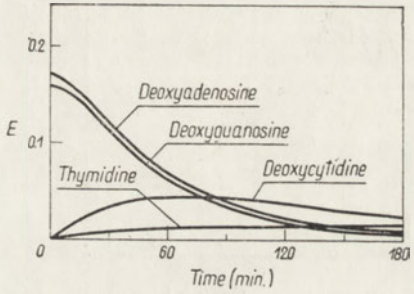

Fig. 10

Fig. 9. Kinetic analysis of the destruction of deoxyribose. For significance of E_0 and E_∞, see text

Fig. 10. Influence of hot PCA on the extinction of free deoxyribosides as a function of time. The curves shown are calculated on the basis of experimental determinations for 0.025 μmole per ml. of each nucleoside, i.e. approximately the same concentrations as in the experiments illustrated in Fig. 11

but as time passes the extinction increases. For deoxycytidine the increase is substantial, after 3 hr. this substance gives greater extinction than the other three substances together.

The behaviour of a mixture of all four deoxyribosides was then studied. It is easy to see that addition of the four curves must give a gradually decreasing curve originating in a point equal to the sum of the purine nucleoside extinction. There are two reasons for believing that this composite curve also follows a first order scheme. The first one is that it can be shown mathematically to be exponential, as a first approximation. The second one is that the curve for deoxycytidine very much resembles the curve arising as the differences between two first order curves (cf. Fig. 12). The rate constant of the composite curve will of course be lower than that of the purine nucleosides.

The results of an experiment on such a mixture are shown in Fig. 11. It is seen that the first order curve makes an excellent fit. For the curve shown in the Figure the following values were found: $E_0 = 0.389$; $E_\infty = 0.009$ and $k = 0.0050$. The concentration of each pyrimidine nucleoside was 0.025 μmole per ml. The total

concentration of purine deoxyribosides was 0.0475 μmole per ml., thus the extinction per μmole 8.19. From this value it may be concluded that, as a very close approximation, the extrapolated value of E_0 represents the extinction of the purine deoxyribose present.

It will be noticed that all the values of k obtained with various types of DNA, except the one for rat liver DNA, lie between the values for the purine nucleosides and the value for the mixture. These values were all obtained in flasks and therefore, as will be discussed below, they will be somewhat lower than when the extraction is carried out in tubes. When tubes are used, the k for rat liver DNA will also fall within the range mentioned above. An explanation of this finding might be

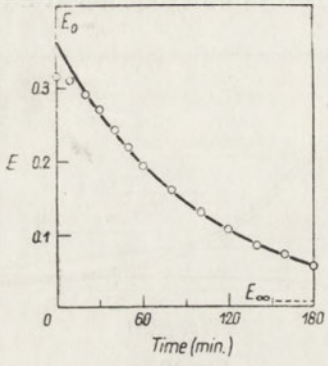

Fig. 11

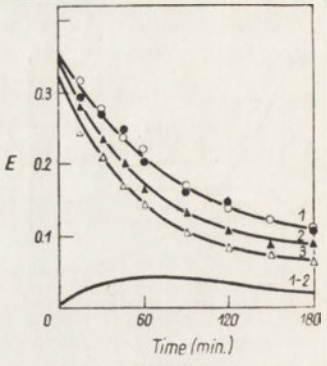

Fig. 12

Fig. 11. Kinetic analysis of the destruction of deoxyribose in a mixture of four deoxynucleosides. For significance of E_0 and E_∞, see text

Fig. 12. Extraction of rabbit liver powder with hot PCA. Influence of reaction vessel and of centrifugation on the extinction obtained with diphenylamine. (●), Flask, centrifugation; (○), flask, no centrifugation; (△), tubes, centrifugation; (▲), tubes, no centrifugation

that the curves for DNA essentially represent purine deoxyribose destruction, but that the rate of this process is partly reduced. It is more likely that the curves represent both purine and pyrimidine deoxyribose destruction, the lower constants thus resulting from a higher degree of interference by pyrimidine deoxyribose. If this latter explanation is correct, it will be realized that, opposite to what would normally be presumed, a high constant corresponds to a greater stability of the DNA molecule.

For the practical utilization of the present findings for DNA analysis it is fortunate that the extrapolated E_0 value, as shown above, is little influenced by the presence of pyrimidine deoxyribosides.

Another interesting point is observed when the curve for kidney DNA (Fig. 8) is compared with those for the low-molecular deoxyribose compounds (Fig. 9 and 10). It will be seen that maximum extinction, as indicated by the curve, is reached at a much earlier time in the former case. The activation necessary for deoxyribose to react with diphenylamine apparently may occur much faster when it is bound in DNA.

The extinction of DNA

The mean of the four values of extinction per μmole deoxyribose reported in the preceding section is 8.34. Mammalian DNA contains 21% of guanine and cytosine, 29% of adenine and thymine. The average molecular weight of a bound nucleotide in DNA of this composition is 309. The content of purine deoxyribose in 309 μg. is thus 0.5 μmole, the expected extinction per mg. DNA therefore $8.34 \times 0.5/0.309 = 13.5$. Many determinations of this value have been made for high-polymeric preparations of DNA. The DNA content used in these calculations has been based on determinations of the P content, corrected for RNA when present.

In connection with the work previously published it was observed that the values tended to decrease slightly as the age of the DNA solution increased. An extrapolation to zero time gave a value of 12.1 for both calf thymus and salmon sperm DNA. This value is probably more accurate than any value arrived at from an experimental standard. We have therefore used this value in the calculations, and a standard DNA has been included in our experiments merely to check the experimental procedure.

In later work, including the experiments on the low-molecular deoxyribose compounds, the extinction values have been slightly higher. Thus we have found a mean value of 13.2 (standard error ± 0.1) for our DNA preparations. The agreement between this value and the theoretical value is very satisfactory. Since the errors involved in calculating the DNA content from P determinations must be rather large, it seems likely that the latter is more correct, and we have therefore lately used the value 13.5 in our calculations. Our results stress the fact that this value is not a natural constant, but is subjected to variations, and it is likely that it may vary somewhat from one laboratory to another, depending on the chemicals used, etc.

The prerequisite for using this value for DNA extracted from tissue powder is of course that the same agreement between expected and observed extinction holds also for these types of DNA. This question can only be answered by comparing the results of DNA analyses obtained with two independent methods, as will be done in the following section.

Comparison with a microbiological method

Two possibilities exist when the results of DNA analyses obtained according to the present principles are to be compared with another DNA estimation method. These are, either to use one of the other chemical methods, measuring the P content or the UV absorption, or else a different method, preferably specific for deoxyribose. Due partly to the various objections against the chemical methods, and partly to the fact that a microbiological method is available in our laboratory, the latter course was taken. The microbiological DNA assay has been developed

by Hoff-Jørgensen [9, 10]. This method is based on the fact that the supply of deoxyribosides is a nutritional requirement of *Thermobacterium acidophilum R 26*. Samples of DNA must be enzymically digested before assay. The growth response is measured by turbidimetry, but may also be determined by titration of the lactic acid formed (Løvtrup & Roos [14]). The method as used in our laboratory has been modified in several ways (Løvtrup & Roos [13, 15] and Løvtrup & Roos, in preparation).

Table 1

Chemical and microbiological determination of DNA from tissue dry powders

Dry powders were obtained from 20% tissue homogenates extracted with an equal volume of 10% perchloric acid, twice with ethanol, twice with ether and dried at room temperature.

Tissue powder	Chemical (mg. DNA/100	Microbiological mg. dry powder)	Chemical/Microbiological
Calf thymus	17.3	17.6	0.98
Rat liver	1.04	1.10	0.95
Rat kidney	1.76	1.67	1.05
Rat heart	0.63	0.61	1.03
Rat brain	1.01	1.04	0.97
Rabbit liver	0.93	1.01	0.92
Rabbit kidney	1.69	1.72	0.98
Rabbit heart	0.85	0.90	0.94
Rabbit brain	0.61	0.59	1.04
		Mean	0.986 ± 0.046

In Table 1 are presented the results obtained on dry powders of calf thymus and of liver, kidney, heart, and brain from rat and rabbit. The latter results with the chemical method are those published previously [16], but corrected by introduction of the conversion factor as discussed above. The ratios of chemical to microbiological determinations (C/M) show that the yield is almost the same. Microbiological assays are known to possess less precision than chemical methods. Without entering a discussion of this point, which will be done elsewhere, it may be stressed that most of the C/M variation may be reasonably ascribed to the microbiological method.

Procedure for chemical determination of DNA

A procedure for chemical determination of DNA was suggested in the previous publication [16] but due to later experiences certain modifications have been introduced. The method is based on a knowledge of the kinetics of the reaction through which the extinction of the diphenylamine reaction disappears, i.e., the destruction of

deoxyribose. In the first paper, in order to study this reaction a large sample of DNA or tissue powder in 5% PCA was heated at 90°, in an Erlenmeyer flask, the samples for analysis being withdrawn at various intervals of time. For routine DNA determinations such kinetic analysis is unnecessary. Under these circumstances heating of smaller samples in smaller containers, e.g., test tubes or centrifuge tubes, would appear more expedient. When smaller extraction volumes are used, it becomes rather difficult to withdraw samples free of tissue powder, and a preliminary centrifugation is therefore indicated. When these modifications were introduced, we invariably found lower E_{60} values in tubes than in flasks. Two reasons were found to account for this. When results in tubes, with and without centrifugation were compared, a constant difference in extinction was found throughout the course of the experiment (cf. curves 2 and 3 in Fig. 12). There is thus a fraction reacting with diphenylamine, which is not changed by treatment with hot PCA, and which remains undissolved. When uncentrifuged tubes and flasks were compared, it was found that the rate constant in tubes was higher than in flasks. In the former case the constant is approaching the one for purine deoxyribosides, in the latter case the constant is closer to that for a mixture of purine and pyrimidine nucleosides, but the extrapolated E_0 values are approximately the same in both cases (Fig. 12, curves 1 and 2). The differences between these curves (1 - 2 in Fig. 12] plotted on the diagram give a curve closely resembling the one for deoxycytidine (Fig. 10). We have tried in vain to account for this difference (Løvtrup & Roos, in preparation).

This observation does not invalidate the method, but it will be realized that correct results can be obtained only when the kinetic analysis is carried out under conditions identical with those employed for determination of DNA in unknown samples.

Extraction. For work with solutions of high-polymeric DNA we have adopted the following procedure: 150 μl. of DNA solution in 0.01 N-NaOH (1 mg. per ml.) is pipetted into a test tube together with 2.85 ml. PCA adjusted to make the final concentration 5% (0.5 M). After heating at 90° for a specified time, two samples of 1 ml. are added to other test tubes for colorimetric determination.

When dry powders are used, 1 - 10 mg. powder — depending on the DNA content — is weighed into a tube, and extracted with 5 ml. 5% PCA. As described previously [16], lipid extraction is not necessary in the present method. In routine work it may therefore be possible to use homogenates, either directly or after extraction with cold PCA. Unknown samples, for which the kinetics is known, are incubated only for one period of time, which may be adjusted in accordance with the type of tissue investigated. Certain kinds of DNA, e.g. kidney and thymus DNA, are easily extracted, and 20 - 30 min. heating time is sufficient for complete extraction. Other types, especially liver DNA, require one hour of extraction. After incubation, samples are withdrawn as above. To avoid evaporation, tubes must be stoppered during incubation. Whatever source of DNA is used, the amount should be adjusted so that the DNA content at zero time is 25 - 50 μg. per ml. PCA.

Colorimetry. To the 1 ml. extract samples is added 2 ml. of reagent (1.5 g. diphenylamine and 1.5 ml. conc. H_2SO_4 in 100 ml. glacial acetic acid to which 0.5 ml. 1.6% acetaldehyde [2] is added immediately before use). Incubation at least 15 hr. at 30°. The extinction is read in 1 cm. cuvettes at 600 mμ. As appears from the discussion in a previous section it seems questionable whether the use of a DNA standard is of advantage in the present method.

Calculations. The mathematics involved in the calculations of the parameters is very simple. When both E_0 and E_∞ are unknown, k is most accurately estimated by the principle of Guggenheim [8]. This procedure may be applied in the following way: A smooth curve is drawn through the experimental points, and from this the extinction values E_{10}, E_{20}, E_{30}, etc. are estimated. The logarithm of the difference between pairs of values separated by equal periods of time are calculated [e.g. log $(E_{10} - E_{70})$, log $(E_{20} - E_{80})$, etc.]. These values are plotted as ordinates in a diagram against time (10′, 20′, etc.). The slope of the resulting straight line equals k. It should be recalled that the earliest experimental values for certain types of DNA may deviate considerably from the first order curve. Likewise, a very small error in the drawing of the last part of the curve may give rise to considerable deviations from the straight line mentioned. When deviations occur it may often be possible to get a good estimate of k by drawing the line through the middle points only, in other cases it may be necessary to redraw the curve.

For the calculation of E_0 and E_∞ two points on the curve are selected, one near the beginning and one later (e.g., E_{20} and E_{150}). It is important to check that the corresponding values in the diagram lie on the straight line. For the corresponding two values of t the antilogarithms of $-kt$ are calculated. It will be realized from the first order equation $\log \frac{E_t - E_\infty}{E_0 - E_\infty} = -k \times t$ that the ratio between these calculated values equals $\frac{E_{20} - E_\infty}{E_{150} - E_\infty}$. It is thus possible to calculate E_∞. By inserting this in the equation for one of the two selected points $E_0 - E_\infty$, E_0 may be estimated. In order to control that the parameters thus obtained really give a satisfactory curve, values of $E_t - E_\infty$ were calculated as the antilogarithm to the value arrived at by subtracting 10 k, 20 k, etc. from log $(E_0 - E_\infty)$. A wrong estimate of k is easily discovered when the calculated curve is plotted together with the experimental points.

For calculation of the DNA content in unknown samples, the values of k and E_0/E_∞ must be known. One may proceed on the assumption that these parameters are constant for a certain type of DNA, but it is wise to check this point whenever possible. For the unknown sample the value E_t, i.e. the extinction after heating for t minutes should be determined experimentally.

From these parameters E_∞ may be calculated from the equation

$$\log\left(\frac{E_t}{E_\infty} - 1\right) = \log\left(\frac{E_0}{E_\infty} - 1\right) - k \times t$$

E_0 is now obtained from the ratio E_0/E_∞. As discussed above this value is too high, and a correction must be introduced, giving a better estimation of zero time extinction, E_0^*. The calculations leading to this value are quite complicated, but a very good approximation is obtained by subtracting 1% for each 10 min. of the time required for complete extraction, e.g., 6% for liver and 2% for kidney.

It may be of interest to consider the impact on the calculated value of E_0, of errors involved in the determination of the parameters in the equation. By introducing values from actual experiments, it was found that a 5% error in the determination of E_t gives rise to a 5% error in E_0. A 5% error in E_∞ causes an error of 0 - 0.5% in E_0, depending on the numerical value of E_∞. Errors of 5% in k and t give errors in E_0 less than 1%. As far as the velocity constant is concerned, it should be recalled that it is dependent on the temperature. However, if this is carefully controlled, k may be determined with quite high accuracy.

It is thus seen that the precision of the method is almost entirely dependent on the determination of E_t. In order to evaluate the error involved in this determination, the value of E_{60} was determined simultaneously on 10 samples of standard DNA. The found mean extinction was 0.190, standard deviation ± 0.0014, corresponding to 0.7%. With 7 samples of calf thymus powder the result was 0.189 ± 0.0032, relative error 1.7%. It is probable that the error may be larger with powders from which DNA is more difficult to extract.

CONCLUSION

Chemical determination of DNA is no easy task. The main reasons for this are that quantitative separation between RNA and DNA is difficult, that various substances may interfere, and that complete extraction of DNA is difficult to achieve. One or more of these difficulties are encountered in the three most common methods for DNA determination. In the method suggested in the present paper these difficulties seem to have been overcome to some degree. No separation of nucleic acids is involved, the very specific diphenylamine reaction is little influenced by interfering substances, and prolonged treatment with PCA at 90° secures complete extraction. The consequent decrease in extinction is corrected for.

With careful work a satisfactory relative accuracy may be achieved. The question of the absolute accuracy may not be definitely settled, but comparison with a microbiological method suggests that the yield is not much below 100%.

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CHEMICZNE OZNACZANIE DNA W TKANKACH ZWIERZĘCYCH

Streszczenie

Omówiono główne trudności oznaczania DNA w trzech ogólnie stosowanych metodach. Przedstawiono ulepszoną metodę, w której trudności te nie występują, gdyż 1°, nie jest konieczne rozdzielanie kwasów nukleinowych; 2°, bardzo specyficzna reakcja z dwufenyloaminą jest mało wrażliwa na interferujące związki; 3°, całkowita ekstrakcja DNA jest zapewniona przez przedłużone traktowanie kwasem nadchlorowym w 90°. Wskazano również sposób korygowania zachodzącego zmniejszenia ekstynkcji. Osiągana wydajność, w porównaniu z testem mikrobiologicznym, wynosi prawie 100%.

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RECENZJE KSIĄŻEK

PLAMEŇOVÁ FOTOMETRIA. R. Herrmann. Tłumaczył z niemieckiego K. Marcinka. Bratislava. Slovenské Vydavatel'stvo Technickej Literatury. 1961. 308 str. Cena 30.50 koron czeskich.

Monografię można podzielić na cztery części. W pierwszej części omówiono takie teoretyczne zagadnienia odnoszące się do fotometrii płomieniowej jak powstawanie płomienia, jego właściwości, zachowanie się atomów w płomieniu itd. Druga część obejmuje opis aparatury do utrzymywania płomienia i dowozu badanych substancji, optykę i elektronikę, fotometrię przy użyciu filtrów, spektrofotometrię i spektrografię. W trzeciej części omówiono pewne problemy związane ze stosowaniem metody w praktyce, np. kalibrowanie, czułość metody, sposoby unikania błędów itd. W czwartej części podano przegląd zastosowań, uwzględniono przy tym zagadnienia związane z rolnictwem, botaniką, analizą środków spożywczych, zoologią, medycyną (specjalne rozdziały poświęcono oznaczaniu sodu, potasu i wapnia w płynach ustrojowych), farmacją, przemysłem chemicznym itd. Książkę zamyka lista widm i obszerna literatura przedmiotu: 602 pozycje oryginału niemieckiego i 583 pozycje dodane przez tłumacza, który uwzględnił te pozycje piśmiennictwa, które ukazały się od 1956 roku, czyli od daty wydania niemieckiego. Dodane pozycje obejmują także prace radzieckie, węgierskie, polskie, czeskie i słowackie.

Książka może z powodzeniem służyć jako przewodnik w tej coraz bardziej potrzebnej metodzie analitycznej.

Tadeusz Korzybski

INTRODUCTION TO ELECTRON MICROSCOPY. Saul Wischnitzer. Pergamon Press, Oxford, New York, London, Paris 1962. Stron 132. 42s.

Używanie mikroskopu elektronowego wymaga znajomości podstaw optyki elektronowej, znajomości konstrukcji samego aparatu i metod przygotowywania materiału do badań. Książka Wischnitzera poświęcona jest w zasadzie dwom pierwszym zagadnieniom. Przedstawianie sposobów przygotowywania materiału do badań wymaga w wielu przypadkach specjalnego opracowania, z uwagi na różnorodność i specyfikę badanego materiału jak również z uwagi na oczekiwaną odpowiedź. Autor nie omawia tych zagadnień odsyłając w tych przypadkach czytelnika do literatury specjalnej. W tym celu zestawia on literaturę w odrębne grupy zagadnień dotyczących preparatyki ogólnej, cytologii, badań cytoplazmy i organelli cytoplazmatycznych, tkanek nowotworowych itd.

Wychodząc ze słusznego założenia, że każdy kto chce pracować z mikroskopem elektronowym musi opanować podstawy optyki, a w szczególności podstawy optyki elektronowej autor rozpoczyna swój wykład od tych dwóch zagadnień. Omawia kolejno naturę promieni świetlnych, zjawisko dyfrakcji, zasadę mikroskopu optycznego i zdolność rozdzielczą. Następnie przechodzi do omówienia natury promieni

elektronowych, ich emisji i działania pól magnetycznych jako soczewek. Tę część wykładu kończy omówieniem soczewek elektrostatycznych i bardzo pomysłowo przedstawionym na rysunku porównaniem zasady działania mikroskopu optycznego i mikroskopu elektronowego.

Druga część wykładu poświęcona mikroskopowi elektronowemu obejmuje omówienie poszczególnych części mikroskopu elektronowego jak np. katody, soczewek itp., a dalej zasad ich działania i powstawania obrazu. Autor omawia tu również zagadnienia związane z techniką zdjęć.

Całość wykładu rozszerzona jest uzupełnieniami teoretycznymi zjawisk optycznych takich jak aberacja sferyczna, aberacja chromatyczna itp. Uzupełnienia obejmują również omówienie niektórych wyposażeń dodatkowych mikroskopu elektronowego.

Wykład przeprowadzony jest w sposób jasny i przystępny do czego w dużym stopniu przyczyniają się liczne rysunki i wykresy. Przy rozważaniach teoretycznych autor rezygnuje z trudniejszych wzorów matematycznych ograniczając się do podania wzorów najniezbędniejszych, potrzebnych często w praktyce.

Autor osiągnął stawiany sobie cel, dając czytelnikowi zwięzły wstęp do mikroskopii elektronowej. Książkę tę można śmiało polecić każdemu kto zajmuje się lub zamierza zajmować mikroskopią elektronową. Stanowić ona może cenne uzupełnienie podręcznych bibliotek każdego laboratorium mikroskopii elektronowej.

J. W. Szarkowski