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δ-AMINOLEVULINIC ACID DEHYDRATASE FROM PROPIONIC ACID BACTERIA

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δ-Aminolevulinic acid, the precursor of porphyrins and haeme is transformed to porphobilinogen (PBG) by δ-aminolevulinic acid dehydratase (5-aminolevulinate hydrolyase, EC 4.2.1.24). The enzyme was discovered by Dresel & Falk [6]; some of its properties have been described by Gibson *et al.* [8], and at the same time Schmid & Shemin [14] and Schulman [15] published some data concerning this enzyme.

δ-Aminolevulinic acid dehydratase (ALAD) is, as reported by the above mentioned authors, widely distributed in nature and probably present in all aerobic organisms. The enzyme was found in the liver, kidney and bone marrow of mammals. In domestic birds the presence of the enzyme was detected in the erythrocytes of ducks and chickens. It was also found to occur in spinach leaves, brewer's yeast, algae (*Chlorella*), and in the cells of *Bacterium cadaveris*, *Corynebacterium diphtheriae* and *Rhodopseudomonas spheroides*.

Attempts to purify the enzyme from animal material were undertaken by a number of authors. Gibson, Neuberger & Scott [8] isolated ALAD from acetone-dried liver. The purification method was based on ammonium sulphate fractionation, repeated absorption on calcium phosphate gel and elution with buffer solutions. Granick & Mauzerall [9] isolated and purified the enzyme from chick erythrocytes and rabbit reticulocytes by zone electrophoresis after Kunkel. Bogorad & Granick [2] isolated the enzyme from *Chlorella* and Falk *et al.* [7] from spinach leaves.

The present paper describes a method of purification and some properties of δ-aminolevulinic acid dehydratase isolated from propionic acid bacteria, *Propionibacterium shermanii*. The presence of this enzyme has also been demonstrated in the cells of *Propionibacterium petersonii*, *Propionibacterium freudenreichii* and *Propionibacterium arabinosum*.

EXPERIMENTAL

Culture of bacteria and preparation of acetone powder. P. shermanii from the collection in this laboratory (National Collection of Dairy Organisms, no. 839), *P. petersonii* (American Type Culture Collection,

4870), *P. freudenreichii* (A.T.C.C., 6207) and *P. arabinosum* (A.T.C.C., 4965) were grown on a medium containing acid casein hydrolysate, trypsin casein hydrolysate, potassium phosphates, magnesium chloride, ferrous sulphate, calcium pantothenate, biotin and glucose. The bacteria were grown in 10 liter flasks according to the method of Zodrow & Pawełkiewicz [19].

After about 10 days of growth, the bacterial suspension was centrifuged on a Sharples laboratory centrifuge and the acetone powder was prepared after Bartosiński [1]. The bacterial sediment was suspended in a small amount of water and then poured portionwise with vigorous mixing into a twentyfold volume of acetone cooled to about -15°. The mixture was stirred for another 10 min., filtered under reduced pressure through a G3 sintered glass filter, washed several times with cold acetone and dried 24 hr. at room temperature. The bacterial powder obtained could be stored at -10° for several months without losing its enzymic activity.

Chemical synthesis of δ-aminolevulinic acid (ALA). Methyl-5-bromolevulinate (10 g.) obtained from β-carbomethoxypropionyl chloride by Rappe's method [13] was dissolved in 50 ml. anhydrous dimethyl formamide, and 8 g. of solid potassium phthalimide was added [17]. The mixture was shaken for 40 min. at 60°. The phthalyl derivative was extracted with chloroform-water mixture. The aqueous layer was re-extracted with chloroform. The combined chloroform extracts were washed with 20 ml. water, 20 ml. 2 N-NaOH and finally twice with water. The chloroform solution was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was brought to boil in 1500 ml. water and cooled. The obtained methyl 5-phthalimidolevulinate was hydrolysed by boiling for about 10 hr. in 7 N-HCl. The solution was evaporated under vacuum to dryness, the residue dissolved in a small amount of ethanol and recrystallized by adding ethyl acetate. The white crystalline δ-aminolevulinate hydrochloride obtained was further purified by repeated crystallizations from ethanol and ethyl acetate, m.p. 149 - 151° [11].

Calcium phosphate gel. This was prepared by the method of Keilin & Hartree as described by Colowick [3].

Cellulose exchangers. Diethylaminoethyl cellulose (DEAE) and carboxymethyl cellulose were prepared after Peterson & Sober [12].

Chemicals. They were of highest technical purity purchased from Fabryka Odczynników Chemicznych, Gliwice, Poland.

Determinations of proteins and nucleic acids. The measurements of extinction at wavelengths of 260 and 280 mμ according to the method of Warburg & Christian [18] were made in a Uvispeck H 700 spectrophotometer.

Activity of δ-aminolevulinic acid dehydratase. The activity was expressed in μmoles of porphobilinogen formed during 1 hour per 1 mg. of protein. The incubation mixture consisted of 0.2 ml. of the enzyme solution, 0.2 ml. of 0.01 M-δ-aminolevulinic acid hydrochloride adjusted to pH 7.0, 1.0 ml. M/15 phosphate buffer of pH 7.0, and 0.5 ml. $MgSO_4$ solution with 10^{-3} M final concentration. The incubation was carried out for 30 min. on a water bath at 60° since this temperature was found to be optimal for the enzyme. The reaction was stopped by adding 1 ml. of trichloroacetic acid - mercuric chloride solution (80 ml. 5% TCA and 20 ml. 0.2 M-$HgCl_2$) [9]. The mercuric chloride was added to bond the SH groups which interfere with the development of colour under the action of Ehrlich's reagent. After centrifuging off the protein precipitate, 2 ml. of the supernatant was mixed with an equal volume of 4 N-Ehrlich's reagent prepared according to the modification of Mauzerall & Granick [10]. The amount of porphobilinogen formed was determined by measuring the extinction at 552.5 mμ in a Baush & Lomb Spectronic 20 colorimeter, using a solution of reagents as a blank. The amount of porphobilinogen formed in 1 ml. of the examined solution was calculated in μmoles according to Granick & Mauzerall [9] by multiplying the value of extinction obtained in a test tube of 1 cm. in diameter by 0.226.

Enzymic synthesis of porphobilinogen. A mixture of 50 ml. 0.15 M--phosphate buffer of pH 7.0, 25 ml. ALA neutralized to pH 7.0, and 35 ml. partly purified δ-aminolevulinic acid dehydratase (fraction 3) was incubated for 16 hr. in a 500 ml. filter flask under reduced pressure. The temperature was maintained between 38° and 40°. After incubation, 100 ml. of a TCA-$HgCl_2$ solution was added, and the protein precipitate was centrifuged off (10 min. at 4000 *g*). From the clear supernatant PBG was isolated by the method of Cookson & Rimington [5]. For this purpose the solution was adjusted with acetic acid to pH 4.5, and 15% (w/v) mercuric acetate solution was added gradually until the supernatant above the complex of PBG with mercuric acetate gave no reaction with Ehrlich's reagent. The sediment was filtered off, washed with 1% mercuric acetate, suspended in 80 ml. of water and then saturated with H_2S to decompose the mercuric complex. After filtering the HgS sediment, the excess of H_2S was removed by aeration. The solution was concentrated to 25 ml. at room temperature under reduced pressure (to avoid PBG condensation), pH was adjusted with aqueous ammonia solution to 4 and the solution crystallized for about 12 hr. at 0°. The pale pink crystals formed were filtered, washed with water and dried over solid NaOH under reduced pressure. From 42 mg. of δ-aminolevulinic acid 10 mg. of crystalline porphobilinogen, m.p. 184 - 186° (with decomposition) was obtained. Paper chromatography of the crystalline product was carried out after Consden *et al.* [4] in two solvent

systems; it gave one spot with R_F 0.24 in propanol - ammonia - ethyl acetate - acetic acid - water (60:10:25:5:20, by vol.) and one spot with R_F 0.51 in butanol - acetic acid - water (40:10:50, by vol.).

Purification of δ-aminolevulinic acid dehydratase

Acetone powder (10 g.) from propionic acid bacteria was thoroughly ground in a porcelain mortar, then 100 ml. water was added stepwise with stirring. The suspension was left standing for 20 - 30 min. at 0 - 4° and then centrifuged for 20 min. at 34 000 *g*. Concentration of proteins and nucleic acids, and enzymic activity were determined in the supernatant. Further steps of purification were performed at 0 - 4°. The crude extract was treated with solid ammonium sulphate up to 35% saturation and left in a refrigerator for 25 min. The suspension was centrifuged for 20 min. at about 40 000 g. The sediment was discarded, to the supernatant ammonium sulphate was added to 60% saturation, the mixture was left standing in a refrigerator and then centrifuged. The sediment was dissolved in 20 ml. water and the solution dialysed against 2 liter of distilled water. During the 12- or 16-hr. dialysis the water was changed six times until no sulphate ions were detected in the solution. The dialysis residue was adjusted to pH 6.5 with 0.001 M-phosphate buffer and then treated with calcium phosphate gel in the amount of 12 mg. $Ca_3(PO_4)_2$ per 100 mg. protein. After 10 min. the suspension was centrifuged for 5 min. at about 10 000 *g* and the sediment discarded. The protein solution, after being thrice treated with the gel, was chromatographed on a DEAE cellulose column (2 × 15 cm.). The column was prepared as follows. DEAE-cellulose (4.0 g.) was equilibrated with 0.001 M-phosphate buffer of pH 8.15, suspended in water and introduced into the column. After uniform deposition of the exchanger a solution containing about 70 mg. of protein was introduced. Proteins were separated by elution with water, 0.01 M-phosphate buffer of pH 7.2, and further by concentration gradient elution with 0.05 - 0.1 M-phosphate buffer of pH 6.6. Fractions of 10 ml. were collected. The steps of purification are shown in Table 1. The presented data are means from several determinations. δ-Aminolevulinic acid dehydratase present in the extract of *P. shermanii* was purified about 140 times; the specific activity of the enzyme increased from 0.05 in the crude extract to 6.85 (fraction 4a). The separation on DEAE-cellulose gave distinct fractions; the most active were the fractions 4a and 4b. Rechromatography of these fractions on DEAE-cellulose for further purification gave negative results because of the inactivation of the enzyme. As results from the data in Table 1, the first two steps of purification, the fractionation with ammonium sulphate and the treatment with calcium phosphate gel, gave only 6-fold purification, however, the calcium phosphate removed the nucleic acids

Table 1

Purification of δ-aminolevulinic acid dehydratase from P. shermanii

The activity was assayed in a mixture containing: 0.2 ml. enzyme solution; 0.2 ml. 0.01 M-ALA solution; 1 ml. M/15 phosphate buffer, pH 7.0; 0.5 ml. $MgSO_4$ with final concn. 10^{-3} M; and water to 3 ml. Incubation 30 min. at 60°

Fraction	Volume (ml.)	Total protein (mg.)	Activity (μmole PBG/mg. protein)	Total activity	Yield (%)
1. Crude extract	700	11200	0.05	566	100
2. Fraction 35 - 60% sat. $(NH_4)_2SO_4$	80	1920	0.27	528	93
3. Adsorption on $Ca_3(PO_4)_2$ gel (1 - 3×)	130	1196	0.32	384	68
4. Eluates from DEAE - cellulose column					
a) fractions no. 10 - 15	49	3.9	6.85	27	
b) fractions no. 19 - 20	26	6.5	6.42	42	32
c) fractions no. 26 - 30	35	385	0,29	113	

Table 2

Effect of metal ions on the activity of δ-aminolevulinic acid dehydratase

Incubation mixture: 0.2 ml. of enzyme (fraction 4b); 0.2 ml. 0.01 M-ALA solution; 1 ml. M/15 phosphate buffer, pH 7.0; 0.5 ml. metal salt solution, as indicated in the Table to final concn. of 10^{-3} M; water to 3 ml. Incubation 30 min. at 60°

Addition (10^{-3} M)	Activity (%)
Mg^{2+}	100
Cu^{2+}	0
Hg^{2+}	0
Ag^{+}	0
Zn^{2+}	90
Pb^{2+}	60
Fe^{2+}	70
Fe^{3+}	75
Mn^{2+}	90
Ca^{2+}	85
Co^{2+}	90

the presence of which interfered with purification of the enzyme on the ion exchanger. For ammonium sulphate fractionation the reagent of highest grade of purity (Fabryka Odczynników Chemicznych, Gliwice, Poland) was used. Application of ammonium sulphate of lower purity caused inactivation of the enzyme in 90%.

Table 3

Inhibition of δ-aminolevulinic acid dehydratase activity

Incubation mixture as in Table 1, with Mg^{2+} omitted. Fraction 4b of the enzyme preparation was used. Incubation 30 min. at 60°

Addition (10^{-3} M)	Inhibition (%)
Iodoacetic acid	61
6-Chloromercuric-2-nitrophenol	83
EDTA	80
5-Chlorolevulinic acid	28
Tris	46

Table 4

Balance experiment

Incubation mixture as in Table 1. The enzyme after the third step of purification was used. Michaelis constant $K_m = 3.0 \times 10^{-4}$

Incubation time	δ-Aminolevulinic acid (μmoles)	Porphobilinogen (μmoles)
0	3.00	—
1 hr.	1.68	0.64
Difference	-1.32	0.64

Table 5

Enzymic activity of crude extracts from various Propionibacterium *strains*

Protein extract (0.2 ml.) from bacterial acetone powder. Other components as in Table 1. Incubation 30 min. at 60°

Strain	Activity (μmole PBG/mg. protein)
P. shermanii	0.05
P. petersonii	0.015
P. freudenreichii	0.027
P. arabinosum	0.07

Attempts were also made to remove nucleic acids by protamine or streptomycin sulphate precipitation, this method was, however, abandoned since it caused considerable (70%) inactivation of the enzyme. The chromatography on carboxymethyl-cellulose also inactivated the enzyme. Attempts to purify the enzyme by heat denaturation of the contaminating proteins or by fractionation with ethanol at low temperature were also unsuccessful.

The purified enzyme preparation, fractions 4a and 4b, when stored at -10° lost about 10% of its activity after 10 days. The isolated enzyme gave an absorption spectrum characteristic for protein with a maximum

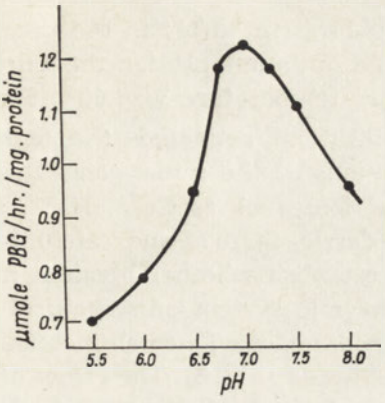

Fig. 1

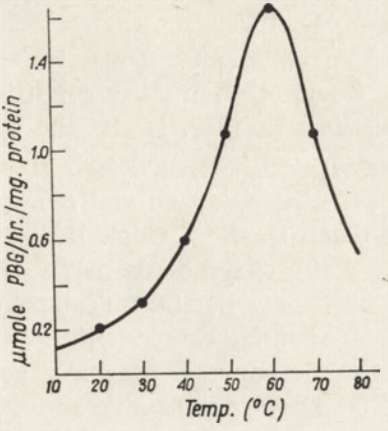

Fig. 2

Fig. 1. Effect of pH on the enzymic activity of purified enzyme preparation. Incubation mixture: 0.2 ml. of the enzyme (0.016 mg. protein), 0.2 ml. 0.01 M-ALA, 0.1 ml. M/15 phosphate buffer, 0.5 ml. $MgSO_4$ solution (final concn. 10^{-3} M), water to 3 ml. Incubation 30 min. at 60°.

Fig. 2. Effect of temperature on reaction yield. Composition of the incubation mixture as given in Fig. 1; phosphate buffer of pH 7.0. Incubation 30 min.

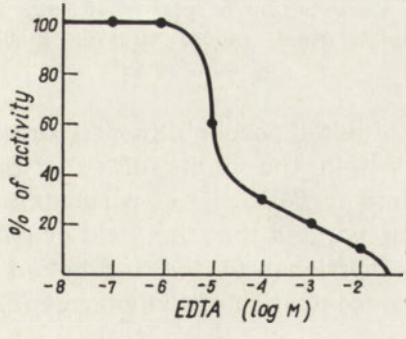

Fig. 3

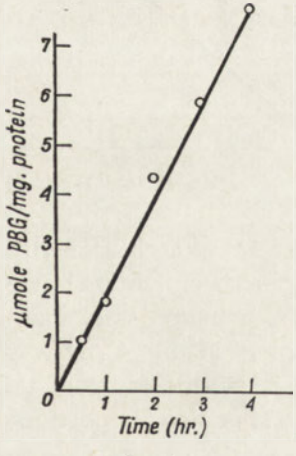

Fig. 4

Fig. 3. Effect of EDTA on enzyme activity. Incubation mixture as in Fig. 1; phosphate buffer of pH 7.0. Incubation 30 min. at 60°.

Fig. 4. Porphobilinogen formation as a function of incubation time. Incubation mixture: 0.1 ml. protein solution (6.3 mg./ml.) after the third step of purification, 0.2 ml. 0.01 M-ALA solution, 1.0 ml. M/15 phosphate buffer of pH 7.0 and 0.5 ml. $MgSO_4$ (final concn. 10^{-3}).

at 278 mμ and a second maximum at 420 mμ. It was repeatedly observed that the enzymic activity of the protein was always associated with its yellow coloration. It would seem that the enzyme contains a yellow pigment. This problem, however, was not studied more extensively in the present work.

The enzymic activity was tested in phosphate buffer in the presence of Mg^{2+} ions. Under these conditions the optimum pH for the purified enzyme was 7.0 (Fig. 1) and the optimum temperature was 60° (Fig. 2).

Magnesium ions at 10^{-3} M concentration activated the enzyme whereas cobaltous, zinc and manganous ions at the same concentration had a much smaller effect. Heavy metal ions such as Cu^{2+}, Hg^{2+}, Ag^{+}, inhibited the enzyme completely while ferric, ferrous and calcium ions inhibited it only partially (Table 2). To establish whether bivalent metal ions are essential components of the enzymic system or structural elements of the enzyme, the influence of sodium ethylenediaminetetraacetate (EDTA) on the enzymic activity was tested (Fig. 3). The effect of the SH group reagents, iodoacetic acid and 6-chloro-2-nitrophenol, is shown in Table 3. It was also found that tris inhibited the enzymic activity by

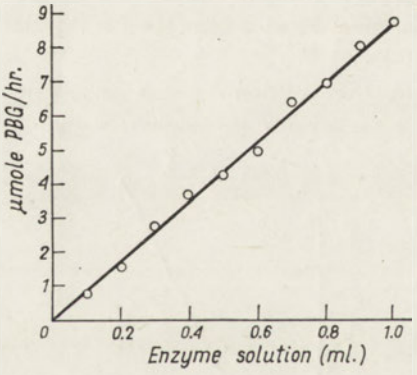

Fig. 5. Porphobilinogen formation at various enzyme concentrations. Incubation mixture: 0.1 - 1.0 ml. enzyme (0.016 mg. protein) solution, 0.2 ml. 0.01 M-ALA solution, 1.0 ml. M/15 phosphate buffer of pH 7.0, 0.5 ml. $MgSO_4$ solution (final concn. 10^{-3} M). Incubation 30 min. at 60°.

a half and that 5-chlorolevulinic acid inhibited porphobilinogen formation. Balance experiments showed that, within the limits of experimental error, from 2 μmoles of δ-aminolevulinic acid 1 μmole of porphobilinogen was formed (Table 4). It was also demonstrated that the yield of porphobilinogen synthesis from ALA is proportional to the incubation time (Fig. 4). It is also proportional to the concentration of the enzyme (Fig. 5).

In acetone-powder extracts of three other strains of propionic acid bacteria the activity of δ-aminolevulinic acid dehydratase was also demonstrated (Table 5). The activity in various preparations of the individual strains was variable and unreproducible depending on the culture, the method of preparation of the acetone powder etc., therefore no conclusion can be drawn as regards the amount of the enzyme in the examined bacteria.

DISCUSSION

Gibson *et al.* [8] demonstrated that δ-aminolevulinic acid dehydratase isolated from the liver is inactivated by heavy metal ions. The same effect on the enzyme from *Propionibacterium shermanii* was observed. Magnesium ion, in contrast to the data reported by Gibson *et al.* for the liver enzyme, was found to activate the bacterial dehydratase. The inhibiting effect of EDTA which was overcome by the addition of Mg^{2+} ions allows to assume that for the full activity of the bacterial enzyme the presence of divalent metal ions, possibly Mg^{2+}, is necessary. It should be noted that Schulman [16] established the presence of magnesium in ALAD preparation isolated from the liver. Inhibition of the activity by iodoacetic acid or 6-chloromercuric-2-nitrophenol, and also by heavy metals, points to the sulphydryl character of the enzyme.

Preliminary experiments indicated that the inhibition caused by these agents was partly overcome by the addition of glutathione or cysteine. These two substances had, however, no influence on the enzymic activity in the absence of the inhibitors. On the other hand, the enzyme isolated from animal tissue by Gibson *et al.* [8] and Schmid & Shemin [14] was activated by glutathione and cysteine.

Between ALAD isolated from *P. shermanii* and that obtained from the liver or erythrocytes there is a difference in optimum temperature for their catalytic activity, which for the bacterial enzyme is 60° while for the animal enzymes it is 38°. The optimum pH for the bacterial dehydratase was found to be 7.0, similarly as for the enzyme isolated by Granick and Gibson.

SUMMARY

1. δ-Aminolevulinic acid dehydratase was found in *Propionibacterium shermanii*, *P. freudenreichii*, *P. petersonii* and *P. arabinosum*.

2. Extracts of acetone-dried *P. shermanii* were fractionated and a 140-fold purification of the enzyme was achieved.

3. The optimum temp. for the purified enzyme was 60° and optimum pH 7.0. The enzyme was inhibited by heavy metals, SH group reagent and EDTA. Magnesium ions activated the enzyme and overcame the inhibiting effect of EDTA.

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DEHYDRATAZA KWASU δ-AMINOLEWULINOWEGO Z BAKTERII KWASU PROPIONOWEGO

Streszczenie

1. Wykazano, że komórki bakterii kwasu propionowego: *Propionibacterium shermanii*, *P. freudenreichii*, *P. petersonii* i *P. arabinosum* zawierają dehydratazę kwasu δ-aminolewulinowego.

2. Ekstrakty z proszku acetonowego *P. shermanii* frakcjonowano i uzyskiwano 140-krotne oczyszczenie enzymu.

3. Optimum temperatury oczyszczonego enzymu wynosiło 60° a optimum pH 7,0. Badaną dehydratazę hamowały odczynniki grup SH oraz EDTA i jony Cu^{2+}, Hg^{2+}, Ag^{+}. Jony magnezu aktywowały enzym a także znosiły inhibicję wywołaną przez EDTA.

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J. KAWIAK

PRESENCE OF CHONDROITIN SULPHATE IN CHONDROCYTES

II. SYNTHESIS OF CHONDROITIN SULPHATE IN THE CELLS *

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The site of mucopolysaccharide synthesis in the connective tissue is discussed by several authors. Most of the autoradiographic observations [18, 6, 3] suggested that synthesis of sulphate mucopolysaccharides occurs in the cells, other authors [19], however, supposed that only precursors for polysaccharide synthesis are produced in the cells and that further synthetic processes go on in the intercellular space.

Histochemical and autoradiographic evidence for the presence of chondroitin sulphate in chondrocytes has been previously described [15]. In the present experiments a more direct approach to the problem was possible because of the use of a micromethod, similar in some principles to the extraction methods for nucleic acids [8, 9]. This micromethod enabled qualitative analysis to be carried out on small samples of cartilage cells free of extracellular material. The results indicate that mucopolysaccharides are synthesized in the cells.

MATERIALS AND METHODS

Special reagents and solutions. Sodium [^{35}S]sulphate, 1000 μc/ml. carrier-free (The Radiochemical Centre, Amersham, England). Chondroitin sulphate containing 2.95% N and prepared by the alkaline method, and bovine albumin (V fraction) used as standards were commercial products (L. Light & Co., Colnbrook, England). Heparin was an old lyophilized preparation obtained from Prof. J. Zweibaum. Dextran was from Pharmacia, Uppsala, Sweden (No. KH 6811). Lyophilized testicular hyaluronidase preparation was purchased from Wytwórnia Surowic i Szczepionek, Warszawa, Poland. Toluidine blue (G. T. Gurr, London, England) was fractionated with $HgCl_2$ by Dr. A. Bilski, and Amido Schwarz 10 B was a product of Fluka AG Chemische Fabrik, Buchs SG.

Tris-glucose-salt solution used as incubation medium contained sodium [^{35}S]sulphate, 4.7 μc/ml., and consisted of 1 part of 0.05 M-tris-ace-

* Part I [15].

tate buffer, pH 7.5, mixed with 9 parts of a solution composed of 8.0 g. NaCl, 0.2 g. KCl, 0.2 g. $CaCl_2$, 0.1 g. $MgCl_2$, 1.0 g. glucose in 1000 ml. of bidistilled water. Collodion solution was prepared as follows: 1 g. of cellulose tri-,tetranitrate dried over P_2O_5 was dissolved in 100 ml. of glacial acetic acid. The solution was stable for about 1 month; afterwards it became yellow and was no longer used.

Cartilage. Distal and proximal cartilaginous epiphyses of femoral bones were taken from 60 Wistar rats 14 to 20 days old and were prepared as previously described [15].

Incubation with [^{35}S]sulphate. Samples of cartilage of proximal and distal epiphyses (approx. 380 mg. wet wt. each) were incubated separately at 37° in 12 ml. of tris-glucose-salt solution containing [^{35}S]sulphate. After 20 min. incubation, the tissue was transferred to 96% ethanol and left overnight. From each sample of cartilage two fragments were taken, passed through 100% ethanol, benzene, and then embedded in paraffin. They were used for autoradiography and isolation of the chondrocytes. The remaining tissue was dried at 105° to constant weight and used for polysaccharides isolation.

Samples of cartilage heated for 5 min. at 100° and then incubated in the medium containing radioactive sulphate were used as controls.

Isolation of chondroitin sulphate from the cartilage. The procedure described by Boström [4] was applied with two slight differences: smaller portions of cartilage were extracted, and centrifugation at 3000 *g* for 30 min. instead of 12 000 *g* for 10 min. was applied. To 48 mg. of dry cartilage 1 ml. of distilled water was added and the suspension was heated for 15 min. at 100°. After cooling to 5°, 1 ml. of 1 N-NaOH was added and the suspension was mixed for 18 hr. at 5°. Then the sample was adjusted to pH 6 with diluted acetic acid, the cartilage was removed, washed with water and washings were added to the extract. The combined solutions were concentrated *in vacuo* to 1 ml. and centrifuged for 30 min. at 3 000 *g*. To the supernatant 3 volumes of ethanol were added. The precipitate formed was sedimented, washed with 80% ethanol and dried. Then it was dissolved in 1 ml. of distilled water, added with 8 ml. of glacial acetic acid and after 2 hr. at room temp. the precipitate was separated, washed with glacial acetic acid and ethanol, dried and dissolved in 5 ml. of distilled water. The solution was adjusted to pH 5 - 6 with sodium carbonate solution, centrifuged for 30 min. at 3 000 *g* and the clear supernatant dialysed overnight at 5° against two changes of distilled water (2 liter each). The dialysis residue was shaken for 15 min. with 0.2 g. of kaolin. The kaolin was removed, the solution was concentrated and the traces of kaolin were separated. From the supernatant, sodium chondroitin sulphate was precipitated with 3 vol. of ethanol containing 20 mg. of NaCl. The precipitate was dried in air and then over P_2O_5 to constant weight. The yield was 7 - 9 mg.

When 10 mg. portions of standard chondroitin sulphate were submitted to the same procedure, the recovery was about 75%.

Electrophoresis. Paper electrophoresis of the isolated chondroitin sulphate was performed on Whatman no. 2 paper in 0.1 M-acetate buffer, pH 5.0, at 5.0 v/cm. and 3.0 mA. The apparatus with platinum electrodes was similar to that described by Wieland [26]. Standard chondroitin sulphate and heparin moved with the relative velocities as observed by Rienits [22]. Dextran, 300 μg., was used to trace electro-osmotic flow, and 4% acid fuchsin solution was used as an indicator for the observation of the supposed velocity of chondroitin sulphate [11]. Aqueous solutions of the tested substances were applied in about 50 μg. amounts.

Chondroitin sulphate and heparin were stained with 0.1% toluidine blue solution in 30% ethanol. The paper was subsequently washed with several changes of diluted acetic acid. Dextran was stained with saturated solution of Amido Schwarz 10 B in methanol - acetic acid mixture (9 : 1, v/v). Then the strips were washed with the same mixture of methanol and acetic acid.

Paper chromatography. This was performed after Kerby [16]. Ascending technique at 5° on Whatman no. 1 paper was used, with a solvent consisting of 1/15 M-phosphate buffer, pH 6.4, and propanol (3 : 1, v/v). Standards of chondroitin sulphate and heparin in amounts of 13 and 60 μg., respectively, were chromatographed simultaneously. When the mixture of the two standards was chromatographed, two spots were obtained, chondroitin sulphate having the higher R_F value.

Treatment with hyaluronidase. Testicular hyaluronidase, 10.5 mg. (about 600 turbidimetric units) was dissolved in 1 ml. of 0.1 M-acetate buffer, pH 4.6, containing 0.45% NaCl and 0.1% gelatine as stabilizer of the enzyme. The preparation of chondroitin sulphate was incubated with the enzyme solution for 48 hr. at 39° under toluene. After 24 hr. incubation the second portion of the enzyme was added. The incubated mixture was placed on Whatman no. 1 paper, dried and stained with toluidine blue. The control without hyaluronidase was treated in the same manner.

Radioactivity measurements. These were performed on isolated from cartilage chondroitin sulphate in infinite thin layer (50 μg./3.14 cm.^{2}) with an end-window Geiger-Müller counter (1.5 mg./cm.^{2}) and in constant geometric conditions. Self-absorption was negligible, but the self-absorption curves nevertheless were prepared. Corrections were made for radioactivity decay and background. A sufficient number of counts was collected to obtain a statistical error lower than 3%.

The radioactivity on chromatograms and electrophorograms was measured with similar end-window G-M counter supplied with a 10 × 3 mm. aperture.

Autoradiograms of 7 μ cartilage sections were prepared with stripping film AR-10, Kodak Ltd. [21].

Extraction of chondroitin sulphate from isolated chondrocytes. All manipulations were carried out at room temperature (18°) except when otherwise stated. The cartilage embedded in paraffin was cut into 10 μ sections which were adhered without use of egg albumin on coverslips (12 × 32 × 0.17 mm.). After removal of the paraffin, the sections were passed through 100%, 96%, and 70% ethanol, then hydrated with 2% Na_2SO_4 solution (w/v) and water. Each preparation was placed in water, and the cartilage was removed with a needle in order to separate the chondrocytes from the intercellular substance [15]. During this procedure the chondrocytes of the opened lacunae adhering to the coverslip remained on the glass while the intercellular substance together with the cells still embedded in it, was removed. The coverslip with the separated chondrocytes was transferred to de Fonbrune oil chamber in which the subsequent stages of the procedure were performed. The micromanipulator of de Fonbrune was used for handling the cells and for directing the micropipettes. The basic procedures were similar to those used in nucleic acid determinations in cells [8, 9].

Chondrocytes taken at random from different regions of cartilage were transported under paraffin oil onto another coverslip. Groups of 20 chondrocytes were collected and after evaporation of water approx. 0.1 mμl. of 0.5 N-NaOH was added to each group of cells. The extraction was performed at 5° under paraffin oil saturated with water and in an atmosphere saturated with 0.5 N-NaOH solution. After 20 hr. the temperature of the oil chamber was equilibrated with room temperature, the extract was removed with a micropipette (5 to 7 μ diameter of the tip) and evaporated on another coverslip in the oil-free compartment of the chamber. The extracted cells were washed twice with water in the following manner. A drop (0.1 mμl.) of distilled water was drawn into the same micropipette and applied on the cells. Thus the remnants of the solution in the pipette are included into the washings. The two washings were placed on the coverslip near to the extract. The dried extract and washings on the coverslip were washed with glacial or 80% acetic acid to neutralize sodium hydroxide, and surplus acetic acid was evaporated.

Subsequently the coverslip with the extract was dipped in collodion solution and dried overnight above $MgSO_4$. The formed membrane was about 0.4 μ thick, as measured with the interference microscope. The preparation was submitted to dialysis for 24 hr. against distilled water at 5°. Autoradiograms of the dialysed cell extracts were made by covering them with AR-10 emulsion and exposing for 4 weeks. Then extracts were stained with 0.01% toluidine blue solution in 0.1 M-acetate

buffer, pH 4.5. The cells remaining after the extraction were autoradiographed and stained in the same manner.

In preliminary experiments approx. 0.1 mμl. volumes of 0.5% standard chondroitin sulphate solution in 0.5 N-NaOH or in water were evaporated on coverslips, washed with glacial or 80% acetic acid and covered with collodion membrane. As checked with phase-contrast microscope, the chondroitin sulphate persisted on the glass after all this procedure.

RESULTS

Sodium chondroitin sulphate isolated from the cartilage was identified by paper electrophoresis, paper chromatography and hyaluronidase treatment.

On electrophoresis, the isolated preparation moved with the same velocity as the standard chondroitin sulphate. The measurement of radioactivity revealed one peak which matched the area exhibiting metachromatic staining properties (Fig. 1). On paper chromatograms, the isolated chondroitin sulphate moved similarly as the standard sample. Radioactivity occupied the area stained with toluidine blue.

Hyaluronidase treatment of the chondroitin sulphate preparation led to the disappearance of metachromasia which was not observed in control incubations without the enzyme. The sensitivity to hyaluronidase indicates that the isolated ^{35}S-labelled polysaccharide is chondroitin sulphate A and/or C.

Table 1

Radioactivity of chondroitin sulphate isolated from rat femur epiphyseal cartilages incubated with [^{35}S]sulphate

For details see Methods

Cartilage	Counts/min./50 μg. dry wt.
Proximal epiphysis	3020
Distal epiphysis	2508
Control (distal epiphysis heated for 5 min. at 100° before incubation)	74

The preparation contained some protein, the amount of which was estimated semiquantitatively with 2% sulphosalicylic acid and by staining the spot on the filter paper with Amido Schwarz 10 B. The results compared with appropriate bovine serum albumin solutions indicated that no more than 0.5% of protein was present in the isolated chondroitin sulphate.

Chondroitin sulphate obtained from proximal and distal epiphyses incubated with [^{35}S]sulphate showed radioactivities as presented in

Table 1. Relative activities remained unchanged after the preparations were passed through Amberlite IRA-400(OH^-) column (15 × 0.5 cm.). However, small amounts of chondroitin sulphate, proportional in all samples, were adsorbed on the resin. Under the same conditions the mixture of 0.4% water solution of non-radioactive chondroitin sulphate and [^{35}S]Na_2SO_4 (465 000 counts/min.) after passing through the Amberlite column showed a background activity of 13 counts/min. It follows that the chondroitin [^{35}S]sulphate preparation did not contain detectable amounts of inorganic [^{35}S]sulphate.

Autoradiograms of cartilage sections showed that the bulk of radioactivity was localized within the cells; in the matrix only traces of radioactivity were found. Chondrocytes used for micro-extractions were microscopically free from intercellular substance; they were radioactive and showed metachromatic reaction with toluidine blue. The alkaline extraction removed the metachromatic as well as radioactive material from the cells.

The extracts of chondrocytes showed the presence of substance(s) with properties of acid mucopolysaccharides. They were soluble in water, virtually insoluble in glacial acetic acid, did not dialyse through the collodion membrane and showed metachromatic reaction with dilute solutions of toluidine blue. Part of the material in extracts exhibited also an ortochromatic reaction. Autoradiograms of the dialysed chondrocyte extracts showed the presence of ^{35}S-labelled substances (Fig. 2). The radioactivity was not due to inorganic [^{35}S]sulphate nor to [^{35}S]phosphosulphate nucleotides [1, 24] since 2% natrium sulphate (non-radioactive) and water treatments applied to the cells before extraction, as well as acetic acid treatment and dialysis of the extracts would have removed them. The radioactivity, however, persisted after all these procedures. It appears then that radioactive sulphur is present in some high-molecular compound(s). This compound was sensitive to hyaluronidase treatment; the extract treated with the enzyme and dialysed exhibited lower radioactivity.

The above results permit to conclude that in the extract from chondrocytes the sulphated acid mucopolysaccharides were present. Probably these are chondroitin sulphates and/or their complexes with some other compounds.

Fragments of the matrix free from cells, extracted and submitted to the same procedure as extracts from chondrocytes, gave a strong metachromatic reaction. Autoradiograms of these extracts were not prepared.

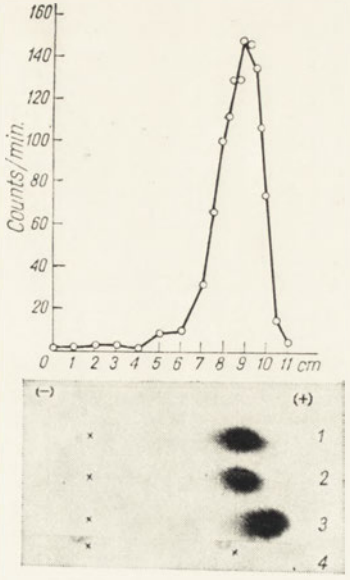

Fig. 1. Paper electrophoresis: (1) standard chondroitin sulphate, (2) chondroitin sulphate extracted from distal epiphysis, (3) heparin, (4) dextran (two small spots). 0.1 M-acetate buffer, pH 5.0 at 5.0 v/cm. and 3.0 mA, 18°. Stained with 0.1% toluidine blue in 30% ethanol; dextran stained with Amido Schwarz 10 B. The diagram represents the radioactivity of electrophorogram of the extracted chondroitin sulphate (2).

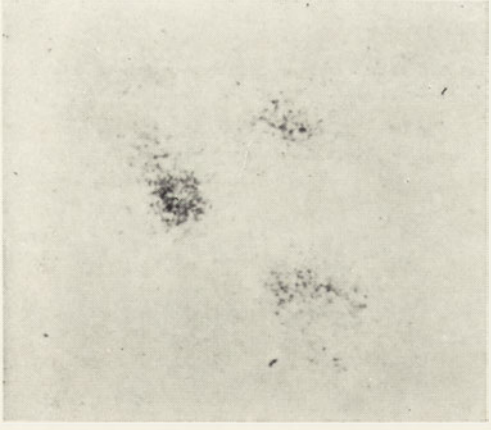

Fig. 2. Autoradiogram of the chondrocyte extract. Twenty cells were extracted with 0.5 N-NaOH for 20 hr. at 5°. The extract and the two washings were put on a coverslip, dried, treated with acetic acid, covered with collodion membrane, dialysed and covered with autoradiographic emulsion. Exposure: 4 weeks. 185 ×.

mucopolysaccharides disappear from the cartilage and probably this leads to increased release of the synthesized polysaccharides from the chondrocytes into the matrix.

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SUMMARY

Possibility of chondroitin sulphate synthesis in cartilage cells was demonstrated. In the cartilage incubated for 20 min. in a medium containing [^{35}S] Na_2SO_4 the radioactivity was localized in the cells. From the cartilage, ^{35}S-labelled chondroitin sulphate was isolated. Separated chondrocytes were extracted with a new micromethod. In the extract, radioactive substances with properties of acid mucopolysaccharides were found.

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OBECNOŚĆ SIARCZANU CHONDROITYNY W CHONDROCYTACH

II. SYNTEZA SIARCZANU CHONDROITYNY W KOMÓRKACH

Streszczenie

Wykazano możliwość syntezy siarczanu chondroityny w komórkach chrząstki. Chrząstka inkubowana przez 20 min. w środowisku zawierającym [^{35}S] Na_2SO_4 miała radioaktywność umiejscowioną w komórkach. Z takiej chrząstki izolowano siarczan chondroityny znaczony ^{35}S. Posługując się nową mikrometodą ekstrahowano chondrocyty izolowane z chrząstki i w wyciągu stwierdzono radioaktywne substancje o własnościach kwaśnych mukopolisacharydów.

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FRACTIONATION OF NATIVE AND HEAT DENATURED TRANSFORMING DNA BY CHLOROFORM TREATMENT

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Since its introduction in 1934 by Sevag [25, 26], the chloroform method has been widely applied to the deproteinization of deoxyribonucleic acids. Its effectiveness in this respect is so well appreciated that, even when other techniques have been used for deproteinization, they are frequently followed by one or two chloroform treatments.

In its usual form the procedure involves the energetic shaking of a saline solution of deoxyribonucleoprotein with one-third to an equal volume of chloroform or a mixture of chloroform and isoamyl or octyl alcohol [3, 25, 26] ostensibly to limit foaming. The protein is denatured by this treatment and following centrifugation of the mixture, is found in the interphase. This treatment has variously been reported to result in a decreased yield of nucleic acid which, in some instances, has been minimized by efficient washing of the chloroform gels [3]. The treatment has been regarded as without any deleterious effect on the nucleic acid itself and this opinion is strongly reinforced by the observation of Avery *et al.* [1] that chloroform deproteinization treatment does not alter the biological activity of T-DNA [1].

It appeared of interest to us to examine the nature of the DNA which has been occasionally reported as appearing in the interphase following chloroform treatment. During such a study it was found that when dilute solutions of DNA are subjected to chloroform treatment, heat-denatured DNA is readily removed from the aqueous phase while native DNA is relatively unaffected. This provides, therefore, an additional method not only for following the course of heat denaturation of DNA but, what is even more important, a simple procedure for the separation of native from heat-denatured DNA. The use of biologically active, such as transforming, DNA offers also the possibility of applying additional criteria to the differentiation of molecules carrying genetic markers.

[1] The following abbreviations are used in this text: T-DNA, transforming DNA; SSC, an aqueous solution of 0.15 M-NaCl in 0.015 M-sodium citrate; A-T, adenine-thymine base pair in DNA; G-C, guanine-cytosine base pair; DHS, C and E refer to the dihydrostreptomycin, cathomycin and erythromycin resistance markers of T-DNA.

During the course of this investigation, a brief communication by Ben-Porat *et al.* [2] reported that, following administration of a pulse of [^{14}C]thymidine to a culture of tissue cells, the newly synthesized labelled DNA could be separated from the total cellular DNA by shaking the crude tissue DNA extract with a mixture of chloroform and isoamyl alcohol, the newly-synthesized DNA passing into the interphase. Notwithstanding the different conditions employed in the investigation of Ben-Porat *et al.* [2], the phenomenon appears of sufficient interest to warrant an investigation of the nature of the fractionation achieved.

In the present study the difference in behaviour towards chloroform of native and heat-denatured DNA was used to follow the course of heat inactivation of streptococcal DNA. One of the objectives was to determine whether partially denatured DNA is due to the total inactivation of only a given fraction of the molecules, with a more resistant fraction remaining unaffected; or to the partial inactivation of all, or most, of the molecules. This was further facilitated by the use of T-DNA.

Particularly interesting is the problem of the residual biological activity of heat-inactivated T-DNA. When inactivation is conducted under conditions such that depurination [10] and chain scission [13, 22, 6] are avoided, the biological activity does not decrease to zero with time of heating, but reaches a plateau value [21] now commonly referred to as the "residual" activity. The question has arisen as to whether the residual activity is an inherent property of the single strands resulting from heat denaturation, or whether it is due to partial renaturation even under conditions where the heated DNA is rapidly chilled [4, 16]. One other possible explanation for this phenomenon may be envisaged, viz. the existence of a fraction so rich in G-C content that the twin strands do not separate under the conditions of heat denaturation hitherto employed. Such a fraction might be expected to retain its biological activity even after heating at 100°. If so, this would indicate that a given marker is distributed amongst different DNA molecules (and this would, in turn, exclude the possibility of concentrating the marker by ordinary DNA fractionation procedures).

From the experiments of Ginoza & Zimm [9] it appears that the residual activity of a pneumococcal T-DNA preparation is quantitatively unaltered when the concentration of the heated DNA is varied over a 100-fold range. This, of course, argues against renaturation as the source of residual activity since renaturation varies, as expected, with the square of the concentration [5, 16].

Density gradient centrifugation has shown that residual activity is located in the denatured DNA band [11, 23]. The question, therefore, poses itself as to whether DNA exhibiting residual activity is in the form of a single strand or a partially dissociated twin strand. An examination of heat-denatured *Bacillus subtilis* T-DNA is claimed to have

demonstrated the existence of a partially denatured fraction with biological activity [17].

It is clear that characterization of the residually active DNA may help to clarify the nature of the heat denaturation mechanism. In this connection an examination has been made of the heat inactivation of T-DNA in sodium perchlorate solution, profiting from the observation [7, 12] that 7.2 M-perchlorate reduces the denaturation temperature of DNA by as much as 40 - 50° as compared to that in SSC. Assuming that no hitherto unknown factors intervene, as regards denaturation of DNA in aqueous perchlorate, then heating of DNA at 100° in 7.2 M-perchlorate should be equivalent to heating at about 140° in SSC. Hence, if a given DNA preparation contains some fraction which is heat-resistant in SSC, it should nonetheless undergo complete denaturation at 100° in 7.2 M-perchlorate since the resultant "equivalent" temperature in the latter solvent is more than sufficient to separate the twin strands of the synthetic polymer poly-dG + poly-dC [15]. If, therefore, the residual activity is linked to some highly enriched G-C fraction, it should be completely inactivated at 100° in 7.2 M-perchlorate.

MATERIAL AND METHODS

The calf thymus DNA, highly polymerized, was prepared according to standard techniques [5a, 13a] in this laboratory. Transforming DNA from *Streptococcus Challis* group H was obtained by a procedure previously described [19] and contained the markers for resistance to dihydrostreptomycin (DHS), cathomycin (C) and erythromycin (E).

Apurinic acid was prepared from calf thymus DNA according to Chargaff [3]. Yeast RNA was a commercial Merck preparation.

Heat denaturation. Samples of 0.5 ml. DNA at a concentration of 20 μg./ml. (unless otherwise stated) were sealed into ampoules which were immersed in a heated water bath for 10 or 15 min. Temperatures were maintained constant to ± 0.15° by means of a Hoeppler ultrathermostat. Immediately after heating the ampoules were removed and dropped into an ice-water bath. Denaturation in aqueous perchlorate was carried out as described by Hamaguchi & Geiduschek [12] using 7.2 M-perchlorate in 0.1 M-sodium versenate.

Measurement of DNA concentration. This was based on readings of extinction at 260 mμ in a Beckman DU spectrophotometer at room temperature. Since mixtures of native and heat-denatured DNA were measured in the same way, this introduces some error due to the hyperchromicity of denatured DNA; streptococcal DNA heated for 10 min. at 100° in SSC, and then rapidly chilled, exhibits 11% hyperchromicity at room temperature. This source of error was disregarded because: (a) it is less than the errors involved in measurement of transforming

activity, and (b) its direction is such that the validity of the final results would be improved if it were taken into account.

Melting curves, or temperature profiles of DNA. These were determined on a Hilger Uvispek or a Unicam SP500 spectrophotometer, using specially constructed compartments for 3 cuvettes, the compartments being maintained at the desired temperature by the circulation of water from a Hoeppler ultrathermostat. The compartment temperature was calibrated against the thermostat temperature by means of copper-constantan thermocouples. Samples were maintained at the desired temperature for 5 min. prior to taking extinction readings so as to allow attainment of equilibrium. No corrections were made for thermal expansion of solvent during heating.

Transforming activity. The procedure for measurement of the activities of the various markers has been previously described [19]. Only the straight line portions of the calibration curves were used.

Chloroform fractionation. To a solution of DNA in a centrifuge test tube was added one-third its volume of chloroform and the tube agitated on a linear shaker with a displacement of 3 cm. and a frequency of 200 cycles/min. Following 20 min. shaking the mixture was centrifuged at 1000 r. p. m. for 2 min. The aqueous phase was removed and submitted to a second chloroform treatment, and this was repeated until the DNA concentration in the aqueous phase had reached a minimum plateau value. Four to five treatments were normally required, the first being always 20 min. and subsequent ones 10 - 15 min. In a typical experiment on a 1 : 1 mixture of native and heat-denatured DNA, 69% remained in the aqueous phase following the first chloroform treatment, 60% following the second and 50% after the fourth.

RESULTS

Separation of native and heat-denatured DNA by means of chloroform. In initial experiments it was observed that shaking with chloroform of a solution of calf thymus DNA in SSC results in the transfer to the interphase of about 30% of the DNA. If the DNA is first heated at 100° and rapidly chilled, then chloroform treatment removes about 60% from the aqueous phase.

For streptococcal DNA the differences were much more pronounced, chloroform treatment removing at most 10% from a native sample and up to 82% after denaturation at 100°.

The foregoing suggested a trial with a 1 : 1 mixture of native and heat-denatured DNA. Following chloroform treatment of such a mixture of streptococcal DNA, about 50% of the total remained in the aqueous phase, suggesting that the native and heat-denatured fractions had been effectively separated. This was further tested by an examination of the

temperature profiles of the DNA remaining in the aqueous phase and that extracted from the interphase. To dissolve the latter fraction, it was suspended in SSC, the chloroform removed at 37° and the resulting precipitate dissolved in SSC and dialysed against the same solvent.

From Fig. 1 it will be seen that the temperature profile of the DNA remaining in the aqueous phase is similar to that for the native DNA. The 2.5% decrease in hyperchromicity as compared to that for the original native preparation, may be due in part to incomplete removal of

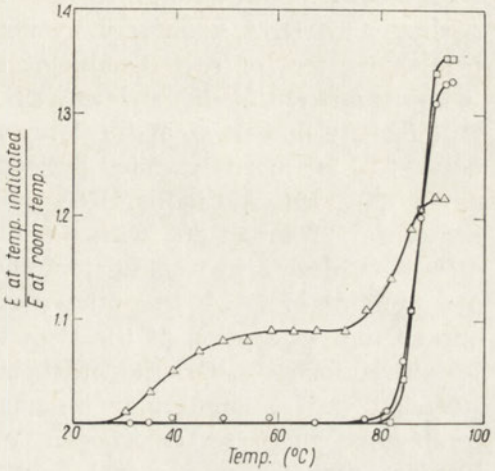

Fig. 1. Temperature profiles in SSC for DNA fractionated from a 1:1 mixture of native and heat-denatured (at 100°) streptococcal DNA. (□), Native DNA; (○), DNA remaining in aqueous phase following chloroform treatment of mixture of native and denatured samples; (△), DNA after heat denaturation carried into interphase by chloroform treatment.

denatured DNA. On the other hand the profile for the DNA extracted from the interphase corresponds to that of a preparation fully denatured at 100°; it is also similar to the profile of a heat-denatured preparation of *Diplococcus pneumoniae* DNA [16].

Influence of addition of isoamyl alcohol. If, in place of chloroform alone, one uses a mixture of isoamyl alcohol - chloroform (1 : 24) such as is normally employed for deproteinization of nucleoprotein [14], the ability to differentiate native from denatured DNA is abolished. Such a mixture will remove from the aqueous phase only a few percent of either native or denatured DNA.

Influence of DNA concentration. While the majority of experiments were conducted with a DNA concentration of 20 μg./ml., identical results were obtained with concentrations as low as 5 μg./ml. Analogous findings prevailed when the DNA concentration was increased to 50 μg./ml. but, at 100 μg./ml., the amount of DNA removed by chloroform treatment was markedly reduced. This is very likely due to appreciable formation of aggregates of denatured DNA formed at these concentrations [24].

Influence of concentration and type of salt. In 0.01 M-NaCl little fractionation of native from denatured DNA could be observed; at best only about 20% of the denatured DNA was removed from the aqueous

phase to the interphase. In 0.15 M-NaCl the results were similar to those obtained with an SSC solution of DNA. In saturated NaCl solution, on the other hand, chloroform treatment removed from the aqueous phase over 90% of either native or denatured DNA. However, from a DNA solution in 7.2 M-perchlorate with added sodium versenate, chloroform treatment removes 9% from a native DNA preparation but only 53% from a denatured sample as compared to 80% and more from an SSC solution.

Effect of depurination. In the above experiments we have been dealing with DNA denatured by heating for short periods at 100°, the principal effect of such treatment being to provoke separation of the twin strands [18, 5]. If, however, DNA is heated at a temperature below that for the mid-point of the temperature profile, i.e. at a temperature below T_m, for more extended periods, the major effect is partial depurination [6, 8, 10]. A Challis DNA sample in SSC (T_m 87°) was therefore heated for 20 hr. at 80° with a resulting decrease in biological activity of 94%. Chloroform treatment of such a solution removed only a minimal amount of DNA from the aqueous phase, from which it would appear that separation of the twin strands is a prerequisite for chloroform fractionation. On the other hand, apurinic acid, which is single stranded with a molecular weight of the order of 15,000 [3], was completely inert to chloroform treatment. Yeast RNA in SSC was likewise resistant to removal from the aqueous phase by chloroform. The effect of chloroform treatment on heat-denatured DNA in SSC consequently appears to be a relatively specific phenomenon.

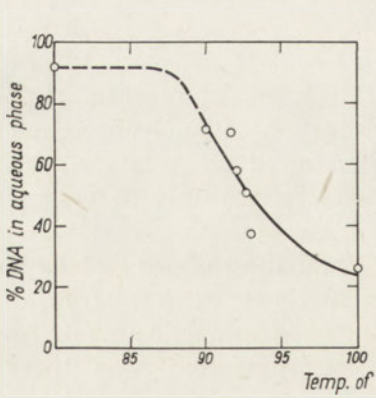

Fig. 2

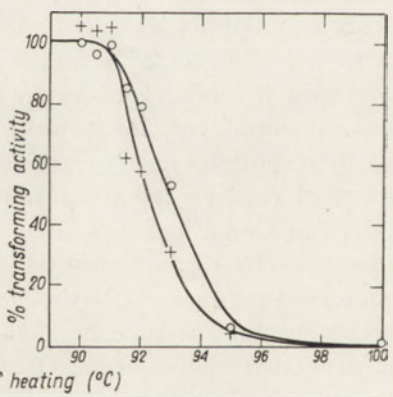

Fig. 3

Fig. 2. The amount of DNA left in aqueous phase after heating at temperature indicated, followed by chloroform treatment to remove heat-denatured DNA (see text for further details).

Fig. 3. Heat inactivation in SSC of streptococcal T-DNA markers at various temperatures. (+). Dihydrostreptomycin resistance marker; (○), cathomycin resistance marker.

Fractionation of partially denatured DNA. Samples of DNA were heated for 10 - 15 min. at various temperatures and then submitted to chloroform treatment. Fig. 2 shows that the amount of DNA removed from the aqueous phase increases with temperature of heating, in accordance with expectation.

Now if heat denaturation of DNA were an "all-or-none" process [20, 4], then in a partially denatured sample we would expect dissociation primarily of the twin strands with a low G-C content, the remainder of the molecules being less affected. Following chloroform treatment such a solution should retain in the aqueous phase a fraction with a higher average G-C content, hence with a higher T_m, than the initial solution. A DNA solution was therefore heated at 91.2°, following which chloroform treatment removed 38% of the DNA from the aqueous phase. However, the temperature profile of the DNA remaining in the aqueous phase exhibited the same T_m and the same breadth as the original unheated sample; the only difference was a 4.5% decrease in hyperchromicity (cf. Fig. 1).

Biological trials

The behaviour of heat-denatured DNA versus chloroform was now extended to follow the inactivation of genetic markers in T-DNA. Transforming activity of the markers was estimated, following heat inactivation, prior to and following chloroform treatment. Fig. 3 exhibits the rates of inactivation of the DHS and C markers in Challis DNA. It will be seen from the figure that the course of inactivation is gradual and proceeds over a range of several degrees. Inactivation begins for each marker at a characteristic temperature and the activity decreases

Table 1

Influence of chloroform fractionation on the specific activity of Challis T-DNA dihydrostreptomycin resistance marker following heat inactivation at various temperatures

Inactivation was carried out for 15 min. For details see text.

Inactivation temp. (°C)	Specific activiiy of unfractionated solution (%)	Following chloroform fractionation	
		DNA remaining in aqueous phase (%)	Specific activity (%)
Room temp.	100	—	—
90.0	91	73	100
91.0	35	52	52
92.1	14	32	35

with increase in temperature to a residual level of approximately 0.5 - 1.0% (see below), which is unaltered even when the temperature is raised to 100°.

Control experiments showed that chloroform treatment of native DNA did not affect or, at most, only slightly decreased the specific activity of a given marker. For a mixture of native and heat-denatured DNA, chloroform treatment provoked a 10 - 20% decrease in specific activity.

Table 2

Influence of chloroform fractionation on the relative specific activities of Challis T-DNA dihydrostreptomycin and cathomycin resistance markers following heat inactivation at various temperatures

Inactivation was carried out for 15 min. For details see text.

Inactivation temp. (°C)	Specific activity of unfractionated solution (%)		Following chloroform fractionation		
	DHS marker	C marker	DNA remaining in aqueous phase (%)	Specific activity of DHS marker (%)	Specific activity of C marker (%)
90.2	28.5	22.3	46	25	32
90.95	8.7	24.5	34	15	30
91.5	7.1	18	30	17	35
92.1	3.6	5.5	22	14	12
92.65	1.8	1.4	19	6.3	7.4

The behaviour of the DHS marker was then followed as a function of temperature of heating, with results shown in Table 1. It will be noted that under these conditions there is a definite increase in the specific activity of the marker following chloroform treatment. The results for another experiment, in which the inactivation of both the DHS and C markers was carried to a level of about 1.5%, are exhibited in Table 2. The increases in specific activities of both markers are here much more marked.

It must, on the other hand, be conceded that the increases in specific activity are not nearly as pronounced as might be expected from the amount of DNA removed from the aqueous phase by chloroform treatment.

Residual activity

Magnitude of residual activity for streptococcal DNA. In order to establish the level of residual activity of heat-denatured DNA, an examination was made of the effect of time of heating at 100° on biological activity. Curve *b* of Fig. 5 (see below) demonstrates the initial,

rapid drop in activity during the first minute, followed by a gradual decrease. On the basis of our present understanding of the nature of the heat-induced modifications in DNA, it seems reasonable to assume that the initial, abrupt, decrease is related to dissociation of the twin strands, and the subsequent gradual loss in activity to depurination [10]

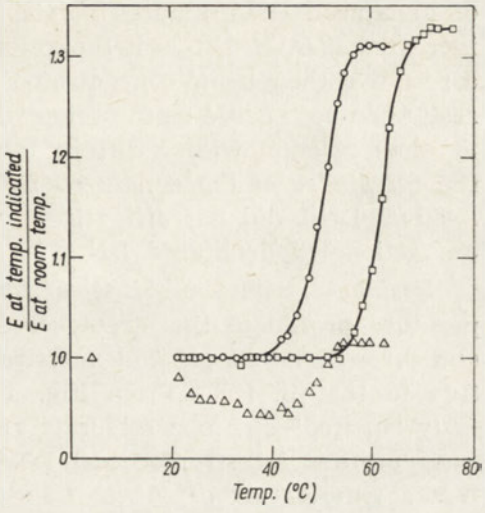

Fig. 4. Temperature profiles of native and heat-denatured streptococcal DNA in SSC and in 7.2 M-$NaClO_4$. (□), Native DNA in 10-fold diluted SSC; (○), native DNA in 7.2 M-$NaClO_4$; (△), DNA heat-denatured (at 100°) in 7.2 M-$NaClO_4$.

and, possibly, some chain scission [13, 22, 6]. From the point of intersection of the extrapolated two portions of the inactivation curve, it appears that the residual activity, corrected for depurination and chain scission, is about 2%, i.e. similar to that obtained for T-DNA from other bacterial systems.

Activity of denatured DNA following chloroform treatment. The purpose of the following experiment was to determine whether the

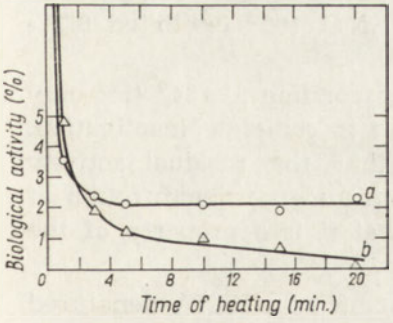

Fig. 5. Course of inactivation with time of T-DNA dihydrostreptomycin resistance marker; (a), at 70° in 7.2 M-$NaClO_4$; (b), at 100° in SSC. Only the lower portions of the inactivation curves are shown, since the initial portions are very steep and practically coincide.

DNA fraction exhibiting residual activity differed from the entire denatured DNA sample as regards its behaviour towards chloroform treatment. A DNA sample was inactivated at 100° to 0.4% residual activity and shaken with chloroform. This treatment resulted in the removal of 72%

of the DNA with no change in the total activity, i.e. the specific activity of the DNA in the aqueous phase increased at least 3-fold. The material in the interphase was washed with physiological saline, the chloroform removed by warming to 37° and the DNA then dissolved in SSC. This solution exhibited about 2% of the activity of the original, native sample. It consequently appears as though chloroform treatment of denatured DNA induces partial renaturation, perhaps by favouring increased contact between separated or partially separated strands, as a result of their being concentrated in the interphase. But the foregoing results do not enable us to decide whether the residual activity is linked to some fraction with a different behaviour towards chloroform than the remainder of the denatured DNA. The temperature profile for the residual DNA did not differ in any marked respect from that for the unfractionated denatured DNA.

Residual activity in 7.2 M-perchlorate. A determination of the temperature profile of the streptococcal DNA in 7.2 M-sodium perchlorate and 0.1 M-EDTA at pH 7 demonstrated a decrease in T_m of 36.5° relative to that in SSC. From Fig. 4 it will be seen that the DNA is fully "melted" out in perchlorate at 58°. A comparison of the temperature profiles for streptococcal DNA in perchlorate and SSC indicates that a temperature of 66° in 7.2 M-perchlorate is equivalent to 100° in SSC, while a temperature of 100° in perchlorate corresponds to 134° in SSC. The latter temperature in SSC is 24° higher than that necessary to completely dissociate the twin-stranded polymer poly-dG + poly-dC [15]. An examination of the biological activity of DNA, which had been subjected to such conditions, was made by heating identical T-DNA samples in 7.2 M-perchlorate at temperatures of 66° and 100°, followed by rapid chilling (see also below and Fig. 5). Subsequent measurements of extinction at 4° showed that both samples exhibited 21% hyperchromicity with respect to the native DNA. That heated at 66° possessed 2.4% residual activity, while the one heated at 100° exhibited 0.7% activity.

Hence even heating to a temperature corresponding to 134° (for only 10 - 15 min., of course) in SSC does not result in complete inactivation. This effectively disposes of the asumption that the residual activity may be linked to some fraction which fails to undergo denaturation at 100° in SSC; but reinforces the argument that it is a property of the dissociated, wholly or partially, twin strands.

It should also be noted that the hyperchromicity of DNA denatured in SSC is 11% and in 7.2 M-$NaClO_4$ is 21 - 22%, with respect to the native DNA at room temperature. Consequently bond reformation during rapid chilling occurs to a markedly lesser extent in perchlorate than in SSC. But the residual activity of DNA denatured in perchlorate is certainly not less than that denatured in SSC. Hence disordered bond refor-

mation, if it does not in fact lower, at least does not increase the activity of denatured DNA.

Finally attention should be drawn to the fact that denaturation in 7.2 M-perchlorate provides a supplementary and quantitatively more accurate procedure for determining the residual activity of a given T-DNA marker. This is so because at 66° in perchlorate, corresponding to 100° in SSC, no depurination [10] or chain scission [13, 22, 6] of the DNA are to be expected. This is fairly well illustrated in Fig. 5, showing the course of inactivation of the T-DNA DHS marker at 70° in 7.2 M-$NaClO_4$ and 100° in SSC. Following the initial rapid decrease in activity in both solvents, that in perchlorate reaches a residual plateau value of about 2%; but in SSC the activity continues to decrease with time, due undoubtedly to depurination and, to a lesser extent, to chain scission.

DISCUSSION

It may be considered as established that chloroform treatment, as described above, provides a relatively simple procedure for the fractionation of heat-denatured from native DNA, whether the criterion applied is biological or physico-chemical. Although application of the method has been largely limited in this study to mixtures of native and heat-denatured DNA, the partial fractionation achieved with unheated calf thymus DNA suggests that its range of application may be considerably broader, as is likewise indicated by the findings of Ben-Porat *et al.* [2].

The conditions for fractionation are, on the other hand, highly specific with respect to several factors: (a) DNA concentration; (b) ionic strength and nature of salt in the aqueous phase; (c) whether the chloroform is used alone or in admixture with an anti-foaming agent. As regards this latter point, it should be noted that the fractionation of newly synthesized DNA achieved by Ben-Porat *et al.* [2] involved the use of a chloroform-isoamyl alcohol mixture, which gives no separation of native and heat-denatured acids.

It remains to determine what are the heat-induced modifications which render the DNA molecules susceptible to removal from the aqueous phase by chloroform. Since depurination induced by heat at neutral pH, which presumably leaves the twin-stranded structure more or less intact [10], is without effect, it would appear that strand separation (whole or partial) is a prerequisite for removal of DNA to the chloroform-water interphase. It then becomes difficult, however, to understand why neither apurinic acid nor RNA are susceptible to chloroform treatment.

Particularly interesting is the fact that if a DNA preparation is fully denatured, as judged by the loss of its characteristic temperature profile, 20% remains resistant to chloroform treatment, i.e. does not pass into the chloroform-water interphase. The reality of this phenomenon is testified to by the fact that 20% remains in the aqueous phase when the initial concentration is varied over a range of 5 to 50 μg./ml. This may be interpreted as indicating that denaturation at 100° results in more than one modification of the native DNA, in other words that denaturation is not an all-or-none phenomenon.

It is, therefore, significant that, following partial heat denaturation of a solution of DNA and removal of the denatured molecules by means of chloroform, the fraction remaining does not exhibit an increase in T_m. In fact, the temperature profile of the remaining fraction is practically identical to that of the original unheated sample, except for a small decrease in temperature hyperchromicity. If the T_m of a given DNA preparation is indeed a linear function of its G-C (or A-T) content, and if denaturation is an all-or-none phenomenon, then those strands with a lower G-C content should melt out first and those remaining in the aqueous phase should exhibit a higher T_m. The absence of such a phenomenon argues against the assumption that heat denaturation of some fraction is due to its lower G-C content. The observed differences in heat resistance may be due to differences in base sequence which do appear to have a pronounced effect on the extent of denaturation as observed following chilling of the heated samples.

Essentially the same conclusion follows from an examination of the effect of heat on the biological activity of transforming DNA. In those experiments where a DNA solution was only partially heat-inactivated and then treated with chloroform, the fraction remaining in the aqueous phase did indeed show an increase in specific activity. But this increase was still considerably less than that expected from the amount of DNA left in the aqueous medium. It, therefore, appears that the biological activity of a partially inactivated marker is a property of molecules which have undergone only partial strand separation.

In all probability molecules with only partially separated twin strands, and therefore not completely denatured, are equally well removed from the aqueous phase by chloroform treatment. It would obviously be highly desirable to examine by some other techniques, such as density gradient centrifugation, the nature of the DNA fractions separated by means of chloroform.

The foregoing conclusion regarding the biological activity of partially denatured molecules is in agreement with the observations of Rownd *et al.* [see 17] on *Bacillus subtilis* DNA and with those of Rownd & Lanyi [see 17] on *H. influenzae* DNA. Our results are in accord with the heat denaturation of DNA as a step-wise phenomenon and suggest also that

the heat inactivation of a genetic marker proceeds likewise in a step-wise fashion.

Insofar as the residual activity of heat-inactivated DNA is concerned, our results indicate that this is not a property of some particular heat-resistant fraction, since denaturation at 100° in aqueous 7.2 M-$NaClO_4$ (which is equivalent to 134° in SSC) does not lead to total inactivation, nor even to a lower residual activity than in SSC. In view of the fact that following chloroform fractionation of DNA inactivated at 100° in SSC, the temperature profile of the aqueous fraction did not differ essentially from that of the entire denatured sample, it seems reasonable to conclude that the residual activity is a property of denatured DNA molecules which underwent strand separation as a result of heating. The results do not, however, permit us to decide whether the residual activity resides in molecules which, following rapid cooling, remain in the form of single strands or of molecules which underwent partial renaturation.

We should like to express our thanks to Professor R. Pakuła for making available the streptococcal strains; Dr. K. L. Wierzchowski for discussions on DNA denaturation; Mrs. A. Golda for technical assistance; and Professor J. Heller for his interest and support in the initiation of work in this field.

SUMMARY

Heat-denatured streptococcal DNA may be separated from native DNA by shaking an aqueous saline solution of the mixture with chloroform and centrifuging. The denatured DNA is then found predominantly in the interphase. The conditions for such fractionation are quite specific with regard to a number of factors such as DNA concentration, ionic strength and type of salt in the medium. No fractionation is observed when the chloroform is used in admixture with an anti-foaming agent such as isoamyl alcohol.

Preliminary observations indicate that both native and heat-denatured calf thymus DNA may be fractionated by the above technique, but the nature of the fractions obtained with native calf thymus DNA remains to be established. Chloroform treatment is without effect on yeast RNA, on apurinic acid, or on DNA heat treated under conditions where only depurination or chain scission take place.

Following controlled partial heat denaturation, chloroform treatment of streptococcal transforming DNA results in the removal from the aqueous phase of only a portion of the DNA. The resultant specific activity of the DNA remaining in the aqueous phase is increased, but not as much as expected.

The overall results are consistent with the view that heat denaturation of DNA, or heat inactivation of transforming DNA, is not an "all-or-none" phenomenon but is rather step-wise in character. The biological activity of a partially inactivated genetic marker is a property of molecules which have undergone only partial strand separation. The residual activity of a heat-denatured transforming DNA is a property of denatured DNA molecules.

Heat denaturation of transforming DNA in 7.2 M-perchlorate provides an excellent quantitative technique for measuring the residual activity of fully heat-denatured DNA under conditions where depurination and chain scission are non-existent.

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FRAKCJONOWANIE RODZIMEGO I CIEPLNIE ZDENATUROWANEGO TRANSFORMUJĄCEGO DNA

Streszczenie

Cieplnie zdenaturowane transformujące DNA paciorkowców można oddzielić od rodzimego DNA przez wytrząsanie z chloroformem mieszaniny takich preparatów w wodnym roztworze soli. Po odwirowaniu zdenaturowane DNA znajduje się w interfazie. Przy takim frakcjonowaniu muszą być zachowane odpowiednie warunki stężenia DNA, siły jonowej roztworu i rodzaju soli w środowisku. Dodanie alkoholu izoamylowego do chloroformu uniemożliwia przejście zdenaturowanego DNA do interfazy.

Wstępne obserwacje wykazują, że przy pomocy powyższej techniki można również frakcjonować rodzime i cieplnie zdenaturowane DNA z grasicy, lecz pozostaje do ustalenia charakter frakcji otrzymywanej z roztworu rodzimego DNA. Traktowanie chloroformem nie ma wpływu na RNA z drożdży, kwas apurynowy oraz DNA ogrzewane w warunkach powodujących jedynie depurynację lub ścinanie łańcuchów.

Traktowanie chloroformem częściowo zdenaturowanego ciepłem transformującego DNA *streptococcus Challis* powoduje usunięcie z fazy wodnej tylko części DNA; specyficzna aktywność transformującego DNA w fazie wodnej wzrasta, lecz w mniejszym stopniu niż można by oczekiwać na podstawie ilości DNA usuwanego z roztworu.

Wyniki doświadczeń są zgodne z poglądem, że denaturacja cieplna DNA, lub inaktywacja cieplna transformującego DNA, nie jest procesem o charakterze „wszystko lub nic", lecz procesem stopniowym. Biologiczna aktywność częściowo zinaktywowanego DNA pochodzi od cząsteczek, których łańcuchy podwójnej spirali uległy tylko częściowemu rozdzieleniu. Resztkowa aktywność cieplnie zdenaturowanego transformującego DNA jest własnością zdenaturowanych cząsteczek DNA.

Denaturacja cieplna transformującego DNA w 7,2 M-nadchloranie sodowym umożliwia dokładny pomiar aktywności resztowej całkowicie zdenaturowanego cieplnie DNA w warunkach, w których nie następuje depurynacja i ścinanie łańcuchów.

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Note added in proof: It has now been found that the degree of fractionation of heat-denatured from native DNA is also dependent on the base composition of the DNA. For DNA with a high G—C content, about 70%, chloroform treatment gives practically no fractionation. Further studies are in progress.

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BASIC PROTEIN FROM MITOCHONDRIA AS INHIBITOR OF THE ENZYMES OF THE ELECTRON TRANSPORT SYSTEM

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Basic proteins, protamine, histones, ribonuclease, lysosyme and the synthetic polylysine, inhibit cytochrome oxidase [10, 6], succinate oxidase and succinate cytochrome c reductase [3, 4], the enzyme systems participating in mitochondrial electron transport.

Previously the isolation from hog kidney mitochondria of a basic protein containing about 25% lysine was reported [9]. The present paper deals with the effect of this protein on mitochondrial electron transport enzymes.

MATERIAL AND METHODS

Mitochondrial basic protein (MBP). This protein was obtained from hog kidney as described previously [9]. It corresponded electrophoretically to the main fraction A of the basic proteins extracted from hog kidney with sulphosalicylic acid [8].

Preparation of mitochondria. Tissues were homogenized in 9 vol. of 0.25 M-sucrose at 0°, the nuclei were sedimented at 600 *g* and the mitochondria were centrifuged at 20 000 *g* for 10 min., then washed twice with, and suspended in, 0.25 M-sucrose solution. One ml. of the suspension contained mitochondria from 1 g. of tissue.

Cytochrome oxidase. The activity was determined in a Warburg apparatus at 37°. Three ml. of the incubating solution contained: mitochondria (0.1 - 0.2 mg. N); cytochrome c, 0.25 μmole; sodium phosphate buffer, pH 7.4, 100 μmoles; sodium ascorbate, pH 7.0, 30 μmoles; and $AlCl_3$, 1 μmole [7]. Liberated CO_2 was absorbed by 0.2 ml. of 10% KOH and oxygen uptake was measured after 10, 20 and 30 min.

Succinate oxidase. The determinations were carried out in a Warburg apparatus at 37°; each vessel contained in 3 ml. volume: mitochondria (0.2 - 0.4 mg. N); cytochrome c, 0.05 μmole; sodium phosphate buffer, pH 7.4, 100 μmoles; sodium succinate, pH 7.4, 300 μmoles; $CaCl_2$, 1 μmole; and $AlCl_3$, 1 μmole [7].

Succinate - cytochrome c reductase. The increase of extinction at 550 mμ (reduced cytochrome c) was measured in 3 ml. of the mixture containing: mitochondria (0.015 - 0.030 mg. N); sodium phosphate buffer,

pH 7.4, 100 μmoles; cytochrome *c* 0.1 μmole; KCN, 3 μmoles; and sodium succinate, pH 7.4, 30 μmoles [11]. The measurements were carried out with a Unicam spectrophotometer in 1 cm. long cuvette every 20 seconds for 3 min. at room temperature varying from 21 to 25° from one experiment to another. The concentration of the reduced cytochrome *c* was calculated in moles by multiplying the extinction values by 5.35×10^{-5} [11].

$NADH_2$ - cytochrome c reductase. The same method was used as for succinate - cytochrome *c* reductase except that 0.3 μmole $NADH_2$ was added instead of sodium succinate [11].

The effect of basic protein. To study the effect of MBP and protamine on the enzymic reactions the protein was added to the respective reaction mixtures before incubation.

Determination of total nitrogen. The nitrogen of mitochondrial preparations was determined by the Kjeldahl method.

Reagents. Cytochrome *c*, Biosedra, France, a solution containing 3 mg. cytochrome *c* in 1 ml.; protamine sulphate, c.p., Biuro Odczynników Chemicznych, Gliwice, Poland; $NADH_2$, Sigma, U.S.A. Other reagents were of analytical grade.

RESULTS

The effect of protamine and MBP from kidney on the activity of the electron transfer enzymes in hog kidney mitochondria is presented in Figs. 1 - 4. For the experiments the same preparation of mitochondria was used. It was found that the inhibiting effect of protamine on succinate oxidase was twice as great as that of MBP; this difference was much smaller for cytochrome oxidase, and nearly the same effect was observed for $NADH_2$ - cytochrome *c* reductase. On the other hand, succinate - cytochrome *c* reductase was affected more by MBP than by protamine.

The inhibitory effect of varying concentrations of MBP and protamine on the enzymic activities is shown in Table 1. It should be noted that the amount of inhibitor producing the maximum of inhibition was equal for protamine and MBP. For cytochrome *c* and succinate oxidases the maximum effects were at over 300 μg./ml. For cytochrome *c* reductases the maxima of inhibition were at over 100 μg./ml. but already at 30 μg./ml. the effect was nearly the same. Basing on these results, in further experiments 500 μg./ml. of basic protein for the oxidases, and 100 μg./ml. for the reductases was used.

The effect of the basic protein isolated from hog kidney mitochondria, on mitochondrial preparations from hog liver and from rat liver and kidney, was studied (Table 2). In each experiment, the results for the two mitochondrial preparations used were virtually the same.

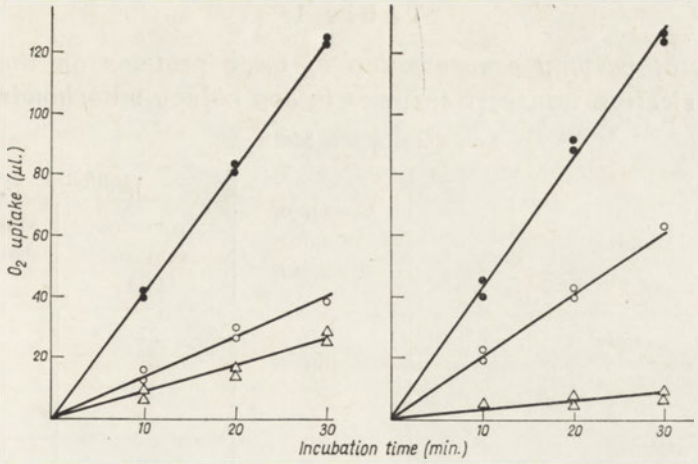

Fig. 1 Fig. 2

Fig. 1. Cytochrome oxidase activity in hog kidney mitochondria: (●), control; (○), with 300 µg./ml. mitochondrial basic protein; (△), with 300 µg./ml. protamine sulphate. Activity was measured by oxygen uptake by mitochondria (0.14 mg. N) at 37°, using sodium ascorbate as electron donor

Fig. 2. Succinate oxidase activity in hog kidney mitochondria: (●), control; (○), with 300 µg./ml. mitochondrial basic protein; (△), with 300 µg./ml. protamine sulphate. Activity was measured by oxygen uptake by mitochondria (0.28 mg. N) using sodium succinate as substrate

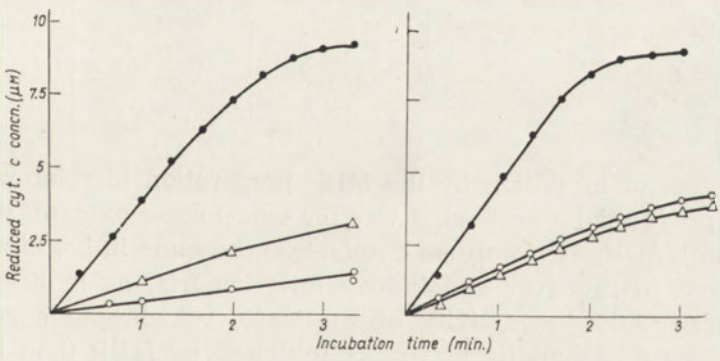

Fig. 3 Fig. 4

Fig. 3. Succinate - cytochrome *c* reductase activity in hog kidney mitochondria: (●), control; (○), with 100 µg./ml. mitochondrial basic protein; (△), with 100 µg./ml. protamine sulphate. Activity was determined at 550 mµ by measuring the cytochrome *c* reduced by mitochondria (0.02 mg. N); sodium succinate was used as substrate

Fig. 4. $NADH_2$ - cytochrome *c* reductase activity in hog kidney mitochondria: (●), control; (○), with 100 µg./ml. mitochondrial basic protein; (△), with 100 µg./ml. protamine sulphate. Activity was determined at 550 mµ by measuring the cytochrome *c* reduced by mitochondria (0.02 mg. N); $NADH_2$ was used as substrate

Table 1

The effect of varying concentration of basic proteins on the activity of electron transport enzymes in hog kidney mitochondria

For details see Methods

Enzyme	Concn. of inhibitor (μg./ml.)	Inhibition by	
		Protamine sulphate (%)	Mitochondrial basic protein (%)
Cytochrome *c* oxidase	10	27	25
	100	65	60
	300	78	70
	600	80	70
Succinate oxidase	10	50	15
	100	80	41
	300	87	54
	600	90	55
Succinate - cytochrome *c* reductase	1	10	25
	3	30	50
	5	37	58
	10	46	71
	15	53	77
	30	64	85
	100	70	90
	150	73	92
$NADH_2$ - cytochrome *c* reductase	5	35	36
	10	45	42
	100	68	65
	150	70	67

The degree of inhibition by the MBP preparation in relation to the inhibition produced by protamine was for cytochrome oxidase, succinate oxidase and $NADH_2$ - cytochrome *c* reductase the same in hog liver mitochondria and in rat liver and kidney mitochondria, as in hog kidney mitochondria. Only the activity of succinate - cytochrome *c* reductase in hog kidney mitochondria was more inhibited by MBP than by protamine; in other mitochondria the effect of both inhibitors was nearly the same, or the inhibition produced by MBP was slightly smaller.

DISCUSSION

The isolation of basic protein from mitochondria and the demonstration of its inhibitory effect on the electron transport system allows to make some suggestions regarding one of the possible mechanisms of metabolic control in mitochondria.

Table 2

Inhibition of some electron transport enzymes by mitochondrial basic protein and protamine, in kidney and liver mitochondria of hog and rat

In experiments on cytochrome *c* and succinate oxidases the concentration of the basic protein was 500 μg./ml. and in experiments on succinate - and $NADH_2$ - cytochrome *c* reductases the concentration of the added basic protein was 100 μg./ml.

Enzyme	Mitochondrial preparation from	Inhibition by			
		Protamine sulphate (%)		Mitochondrial basic protein (%)	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2
Cytochrome *c* oxidase	Hog kidney	80	90	70	80
	Hog liver	85	80	73	75
	Rat kidney	92	80	83	70
	Rat liver	88	85	78	70
Succinate oxidase	Hog kidney	90	90	52	45
	Hog liver	82	81	67	65
	Rat kidney	92	88	65	63
	Rat liver	85	87	42	51
Succinate-cytochrome *c* reductase	Hog kidney	73	75	90	92
	Hog liver	65	75	65	70
	Rat kidney	65	70	51	55
	Rat liver	85	80	60	55
$NADH_2$-cytochrome *c* reductase	Hog kidney	70	75	67	70
	Hog liver	78	80	64	70
	Rat kidney	79	80	73	75
	Rat liver	72	70	70	65

It may be assumed that in mitochondria the basic protein is combined by electrostatic bonds with acidic proteins or phospholipids. Such complexes were demonstrated *in vitro* by Chargaff & Ziff [1] and Nagumo [5]. The mitochondrial basic protein is readily extracted with sulphosalicylic acid which probably dissociates the ionic bonds, and then the protein can pass to the solution; this is similar to the extraction with 0.2 N-H_2SO_4 of histones combined with DNA in cell nuclei. In mitochondria the complex of the basic protein with the acidic component would dissociate reversibly in different sites under the influence of local changes in pH or ions concentration, and would thus regulate the metabolism of the mitochondria.

The mechanism of the inhibitory effect of the basic protein on the electron transport enzymes could be explained by two assumptions. Takemori *et al.* [12] suggested that the inhibition of cytochrome oxidase by basic proteins is due to the combination with the acidic protein and cytochrome *c*. This could be also valid for the components of the Warburg-Keilin system. Machinist *et al.* [3] suggested that the inhibition of

oxidoreduction processes in mitochondria by protamine consists in bonding with the negatively charged pores in the mitochondrial membrane which prevents the entry of external cytochrome *c*. According to both these assumptions, the inhibition by more basic protein should be greater than by less basic protein. In the presented experiments the effect of protamine (80% of arginine [2]) was greater than the effect of the basic protein from hog kidney mitochondria (30% of basic amino acids), with the single exception for hog kidney mitochondria where the succinate - cytochrome *c* reductase was more affected by MBP than by protamine. This could be interpreted as due to a specific effect of hog kidney mitochondria basic protein on the succinate - cytochrome *c* reductase activity in this tissue.

SUMMARY

1. The effect of basic protein obtained from hog kidney mitochondria and that of protamine, on mitochondrial electron transfer enzymes was examined in the kidney and liver mitochondria of hog and rat.

2. Both protamine and mitochondrial basic protein inhibited by 40 - 90% the activities of succinate and cytochrome oxidases as well as succinate - and $NADH_2$ - cytochrome *c* reductases.

3. The inhibition by protamine was more effective than by mitochondrial protein except for succinate - cytochrome *c* reductase in hog kidney.

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ZASADOWE BIAŁKO MITOCHONDRIÓW JAKO INHIBITOR ENZYMÓW PRZENOSZĄCYCH ELEKTRONY

Streszczenie

Zbadano wpływ zasadowego białka z mitochondriów nerki wieprza oraz wpływ protaminy na aktywność oksydazy cytochromowej i bursztynowej, a także na aktywność reduktaz cytochromu *c* zależnych od $NADH_2$ i kwasu bursztynowego w mitochondriach nerki i wątroby wieprza oraz szczura. Zarówno protamina jak i zasadowe białko mitochondrialne hamują aktywność tych enzymów od 40 do 90 procent. W mitochondriach nerki wieprza, zasadowe białko mitochondrialne hamuje mocniej aktywność reduktazy cytochromu *c* zależnej od kwasu bursztynowego niż protamina. Inne enzymy są mocniej hamowane przez protaminę niż przez zasadowe białko mitochondrialne.

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ACID AND ENZYMIC HYDROLYSIS PRODUCTS OF THE CYCLIC PHOSPHATE ESTERS OF 1-(β-D-GLUCOPYRANOSYL)URACIL

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In a previous publication [13] were described the synthesis and identification of the four isomeric monophosphate esters of 1(β-D-glucopyranosyl)uracil, as well as the preparation of the three possible cyclic phosphate isomers which may be formed from these; viz. the 2′:4′ and 4′:6′ with six-membered rings, and the 3′:6′ with a seven-membered ring. The cyclic phosphates are of particular interest from the biological point of view since all three of them were found to be susceptible to ring opening by mammalian tissue enzymes [13]. Additional significance attaches to the cyclic phosphates as a result of the discovery in biological material of adenosine-3′:5′-phosphate and its role in glycogenolysis [7, 6, 10], ketone body formation in liver slices, activation of phosphofructokinase, and other biological functions [10].

Six- and seven-membered cyclic phosphate rings are known to be more resistant to chemical hydrolysis than 5-membered rings [1, 4, 5, 8, 12, 13] and special enzyme systems have been described for the synthesis [10, 11] or hydrolysis [3, 9, 10, 13] of these compounds. In the present communication we describe the products of acid and enzymic hydrolysis of the three cyclic phosphates of glucosyluracil. The latter were obtained by treatment with dicyclohexylcarbodiimide in aqueous pyridine [13] of the 2′-, 3′-, and 6′-monophosphates [1]. For the 2′- and 3′-isomers the only sterically possible products are the 2′:4′- and 3′:6′-cyclic phosphates, respectively. Cyclization of the 6′-monophosphate may, however, lead to the formation of either the 3′:6′-, or 4′:6′-cyclic phosphates, or a mixture of the two. The results of chemical and enzymic hydrolysis, to be described below, show that with the reaction conditions used here [13] the only product is the 4′:6′-cyclic phosphate. This result is all the more striking

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[1] It was previously reported [13] that the cyclization reaction proceeds in 70% yield. We have now found that, if the dicyclohexylcarbodiimide is purified by distillation before use, the yield of cyclic phosphate may be increased to 90%.

in that, under identical conditions, glucose-6-phosphate itself reacts to form a mixture of two cyclic phosphates, presumably the 3:6 and 4:6, both of which are likewise susceptible to enzymic hydrolysis (Żmudzka & Shugar, in preparation).

Acid hydrolysis of the cyclic phosphates was carried out in 1 N-HCl at 100° for 60 min., the samples being contained in sealed ampoules. Under these conditions, hydrolysis proceeded to the extent of about 75 - 80%. The hydrolysis products were identified by paper chromatography in solvents *A*, *B* and *C* (see Table 1); solvent *B* is particularly useful in that it resolves a mixture of the 3′ and 4′ monophosphates from the 2′ and 6′. In addition the hydrolysis products of the 4′:6′-cyclic phosphate (Table 1) were identified by chromatography on a Dowex 2 × 8 formate column previously calibrated against the known monophosphate isomers [13]. This was done by first eluting from the column the three isomeric monophosphates, and then a mixture of the three plus the cyclic phosphate, as shown in Fig. 1.

Table 1

Paper chromatography of 1-(β-D-glucopyranosyl)uracil and its phosphate esters

Whatman no. 1 paper and ascending technique were used. Solvent: *A*, propan-2-ol - ammonia (d - 0.88) - water (7:1:2, by vol.); *B*, sat. $(NH_4)_2SO_4$ - 1 M-CH_3COONa - propan-2-ol (80:18:2, by vol); *C*, methyl cellosolve - methyl ethyl ketone - 3 N-NH_4OH (7:2:3, by vol.)

Compound	R_F in solvent		
	A	*B*	*C*
1(β-D-Glucopyranosyl)uracil	0.38	0.73	—
2′-Phosphate	0.07	0.82	0.18
3′-Phosphate	0.08	0.73	0.19
4′-Phosphate	0.07	0.73	0.21
6′-Phosphate	0.05	0.82	0.16
2′:4′-Cyclic phosphate	0.25	0.57	0.53
3′:6′-Cyclic phosphate	0.25	0.57	0.53
4′:6′-Cyclic phosphate	0.25	0.57	0.53

The fractionation of the products of hydrolysis of glucosyluracil-4′:6′-phosphate (Fig. 2) demonstrates that, apart from a small percentage of 6′-monophosphate, the principal product is the 4′-monophosphate, while the 3′-monophosphate is completely absent. It follows, as noted above, that the product of cyclization of the 6′-monophosphate is exclusively the 4′:6′-cyclic phosphate.

The overall results (Table 2) demonstrate that, with the hydrolysis conditions employed, the 3′:6′-, and 4′:6′-cyclic phosphates are hydrolysed predominantly to products in which the phosphate group remains in a

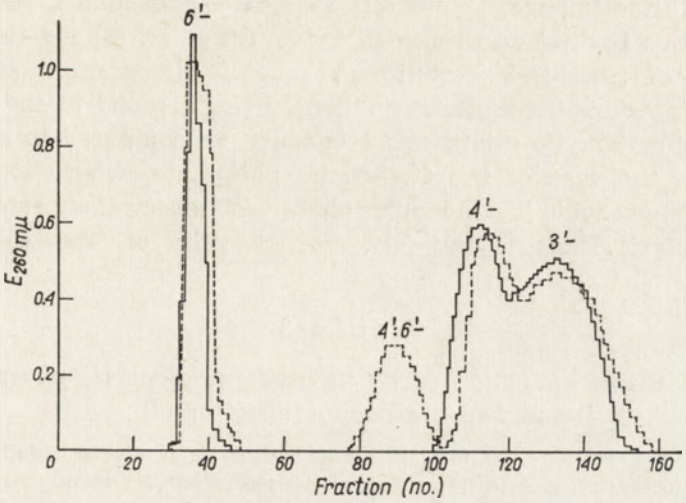

Fig. 1. Column calibration with standard glucosyluracil phosphates: (—), 1.0 mg. each of the 3′-, 4′-, and 6′-monophosphates or (-·-), all the foregoing plus 0.6 mg. of the 4′:6′-cyclic phosphate of glucosyluracil were dissolved in 3 ml. H_2O, brought to pH 9 with 1 N-NH_4OH, and adsorbed on Dowex 2 × 8 formate column (14 × 0.8 cm.). The column was then washed with 300 ml. water and elution carried out with 0.05 M-$HCOONH_4$ - 0.01 M-HCOOH. Fractions of 5 ml. were collected at 10 min. intervals and the extinctions measured at 260 mμ.

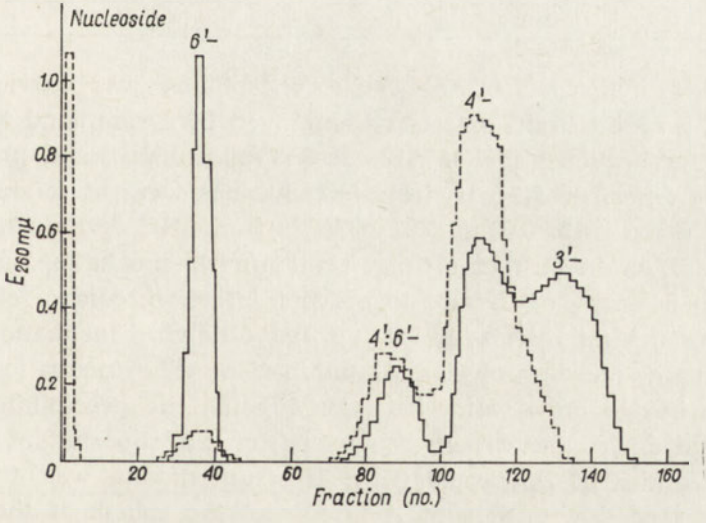

Fig. 2. Analysis of hydrolysis products of glucosyluracil-4′:6′-cyclic phosphate: (-·-), elution pattern for 3 mg. of cyclic phosphate following hydrolysis in 0.2 ml. 1 N-HCl at 100° for 60 min.; (—), elution pattern for mixture of 1.0 mg. each of the 3′-, 4′-, 6′-, and 0.6 mg. 4′:6′-phosphates. Adsorption and elution carried out as described in Fig. 1.

secondary ester linkage, i.e. to the 3′- and 4′-phosphates, respectively. An analogous finding was reported by Smith *et al.* [8] for the alkaline hydrolysis of riboside-3′:5′-cyclic phosphates, the greater stability of a phosphate ester at a secondary linkage being likened to the increased stability of carboxylic esters at a secondary, as compared to a primary, linkage. In the case of the 2′:4′-cyclic phosphate, where each of the two possible products is a monophosphate with a secondary ester linkage, acid hydrolysis leads to the formation largely of the 4′-phosphate (Table 2).

Table 2

Chemical and enzymic hydrolysis products of cyclic phosphates of 1-(β-D-glucopyranosyl)uracil

For hydrolysis conditions see text. Enzymic hydrolysis products usually contained about 20% nucleoside, due to the action of the tissue extract monophosphoesterase. The results represent a typical experiment

Cyclic phosphate	Type of hydrolysis	Monophosphate products and their relative proportions (%)			
		2′-	3′-	4′-	6′-
2′:4′	Chemical	15	—	85	—
	Enzymic	0	—	100	—
3′:6′	Chemical	—	79	—	21
	Enzymic	—	0	—	100
4′:6′	Chemical	—	—	93	7
	Enzymic	—	—	0	100

Enzymic hydrolysis of the cyclic phosphates was carried out by means of a rabbit brain extract as employed by Drummond & Perrott-Yee [3] for decyclization of riboside-3′:5′-phosphates, and prepared as previously described [13]. Hydrolysis conditions were as follows: 0.1 ml. substrate (1 mg./ml.), 0.05 ml. 0.2 M-tris buffer, pH 7.5, 1 µl. 0.1 M-$MgCl_2$ and 0.05 ml. brain extract (14 mg. protein/ml.), incubation for 20 hr. at 37° (in presence of thymol to prevent infection; control of preparation under the microscope preceding and following incubation did not indicate pronounced bacterial contamination). The incubation mixture was then heated on a water bath for 10 min., the precipitated protein centrifuged down, and cations removed from the supernatant by means of a few grains of Dowex 50 (H^+). The supernatant was then paper chromatographed with solvent *A* to control the course of the reaction. The spots corresponding to monophosphates, $R_F \sim 0.07$, were eluted, the eluate concentrated and chromatographed with solvent *B*. For the 4′:6′-cyclic phosphate, the results were checked by column chromatography as described above for acid hydrolysis.

The results for enzymic hydrolysis are collected in Table 2. In the two instances where the cyclic phosphate ring involves both a primary and secondary linkage (i.e. the 3′:6′ and 4′:6′), the hydrolysis products are such that the phosphate group remains involved in a primary ester linkage, hence in a sense inverse to that resulting from acid hydrolysis. However, the enzymic hydrolysis products differ from those obtained by acid hydrolysis in that only a single product is obtained. The action of brain extract is seen from the foregoing results to be analogous to the action of the same extract [3], as well as to the action of the purified phosphodiesterase of Butcher & Sutherland [2], on riboside-3′:5′-cyclic phosphates, the products of which are the 5′-phosphates. The enzyme(s) involved in all the above reactions also require the presence of Mg^{2+} ions for activity [cf. ref. 2, 3].

The foregoing provides no clue as to what enzyme or enzymes hydrolyse the glucosyluracil cyclic phosphates. It is perhaps more than a coincidence that brain extract, which most actively attacks riboside-3′:5′-cyclic phosphates, is also most active against the glucosyluracil cyclic phosphates, although less so than against the riboside cyclic phosphates. It should be recalled that brain extract also decyclizes glucose-4:6-cyclic phosphate [13] and probably glucose-3:6-cyclic phosphate (Żmudzka & Shugar, work in progress). If, therefore, the same enzyme is involved, its range of specificity must be very broad. It is hoped that further work, now in progress, may clarify this problem, as well as the possible metabolic role of the enzyme(s) involved.

SUMMARY

Paper and column chromatographic methods have been applied to the identification of the acid and enzymic hydrolysis products of the three possible cyclic phosphates of 1(β-D-glucopyranosyl)uracil, viz. the 2′:4′-, 3′:6′-, and 4′:6′-phosphates.

Enzymic hydrolysis (by brain tissue extracts) leads to the formation of the 4′-, 6′-, and 6′-phosphates, respectively. Acid hydrolysis of the cyclic phosphates gives a mixture of the two possible monophosphate esters, with one form predominating in each case, viz. the 4′-, 3′-, and 4′-. The results of acid and enzymic hydrolyses are compared and the possible specificity of the enzyme(s) involved discussed.

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PRODUKTY CHEMICZNEJ I ENZYMATYCZNEJ HYDROLIZY CYKLICZNYCH FOSFORANÓW 1-(β-D-GLUKOPYRANOZO)URACYLU

Streszczenie

Zastosowano metody chromatografii bibułowej i kolumnowej do identyfikacji produktów kwasowej i enzymatycznej hydrolizy trzech możliwych cyklicznych fosforanów 1(β-D-glukopyranozo)uracylu, tzn. 2′:4′-, 3′:6′- oraz 4′:6′-fosforanów.

Hydroliza enzymatyczna (ekstraktem z mózgu królika) prowadzi do powstania kolejno 4′-, 6′- i 6′-monoestrów fosforowych. Hydroliza kwasowa daje mieszaninę dwu możliwych monoestrów z wyraźną przewagą jednego z nich; dla kolejnych fosforanów cyklicznych były to 4′-, 3′- i 4′-fosforany.

Porównano wyniki hydrolizy chemicznej i enzymatycznej oraz przedyskutowano możliwą specyficzność rozpatrywanego enzymu(ów).

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ENZYMIC DETERMINATION OF GLUTAMINE WITH RAT KIDNEY PREPARATION

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The available methods for the estimation of glutamine in biological material containing also AMP and amino acids are few and rather inconvenient. The chromatographic methods [16, 13] are rather time consuming, and the methods in which acid or alkaline hydrolysis is applied [14, 3, 12] prior to ammonia or glutamate estimation, are lacking in specificity [9]. The same is true for the ninhydrin-gasometric method of Hamilton [8]. Among the enzymic methods that described by Krebs [10] is highly specific but *Clostridium welchi* used in this method is a very infective material. The Archibald's method for glutaminase preparation from dog kidney [1, 2] is troublesome and the enzyme obtained is not very specific, as ammonia is liberated from glutamine and AMP to the same extent.

In kidney, AMP aminohydrolase is localized in the supernatant fraction [17] and glutaminase in the particulate fraction [7]; therefore it seemed probable that kidney glutaminase may be separated from AMP aminohydrolase and from some other aminohydrolases by centrifugation. This supposition was confirmed. The particulate fraction of rat kidney homogenate suspended in KCN-phosphate solution can be used as a specific enzyme preparation for glutamine deamidation. The liberated ammonia may be estimated directly by microdiffusion if the reaction is performed in a Conway dish.

MATERIALS AND METHODS

Reagents. Glutamine was T. Schuchardt (Görlitz, East Germany) and Fluka (Switzerland) product; 5′-AMP, adenosine, D,L-alanine, L-serine, taurine and L-leucine were L. Light (England) products. Glycine was obtained from T. Schuchardt (East Germany); L-glutamic acid from Merck (West Germany); D,L-threonine from Lachema (Czechoslovakia); L-aspartic acid from Riedel (West Germany); L-asparagine from BDH (England); D,L-lysine hydrochloride and valine from U.S.S.R.; 3′-AMP,

L-alanine and tris from Fluka (Switzerland). Other chemicals were purchased from Gliwicka Fabryka Odczynników, Gliwice, Poland.

Enzyme preparation. Albino rats were killed by a blow on the head and decapitation. The kidneys were removed and homogenized in a Potter-Elvehjem homogenizer with 0.1 M-KCl solution to make a 10% homogenate. The homogenate was centrifuged in cold at 600 *g* for 3 min., the sediment was discarded and the supernatant centrifuged at 24 000 *g* for 15 min. The sediment containing amidohydrolase was washed by suspending it carefully in 0.04 M-KCN solution containing 0.16 M-sodium phosphate, pH 7.7, and centrifuging for 15 min. at 24 000 *g*. The sediment corresponding to 1 gram of kidney was suspended in 3.5 ml. of the KCN-phosphate solution, and used for the assays. The enzyme was stable for about 2 weeks when stored at 1°.

Tissue and blood extracts. Not less than 1 g. tissue or 5 g. blood was dipped into 7 ml. of 15% trichloroacetic acid. While working with the tissues containing glutamine amidohydrolase it was important to put them into acid as soon after removal from the body as possible. The tissue was homogenized in a Potter-Elvehjem homogenizer and the sediment centrifuged off. From the supernatant 5 ml. was transferred into a calibrated test tube, 3 ml. of tris buffer, pH 7.7, containing 50 μmoles sodium phosphate per ml., was added, the solution brought to pH 7.7 with diluted NaOH and made up to 10 ml. with glass-distilled, ammonia-free water.

Glutamine determination. The incubation of the tested sample with the enzyme preparation may be carried out either in the Conway microdiffusion dish [6] or in the microdiffusion bottle described by Brown *et al.* [4]. When Conway technique was used, 1 ml. of the tissue extract was put into a half of the outer chamber, the dish being propped into a sloping position; then 0.3 ml. of the enzyme suspension was added and mixed carefully, and care was taken to prevent the other half of the outer chamber from getting wet.

Next the dish was covered with a dry glass plate and incubated at room temperature (20 - 25°) for 45 min. Then 1 ml. of boric acid with indicator was poured into the central chamber, 1 ml. of saturated Na_2CO_3 solution was added to the incubation mixture and ammonia was estimated by the usual Conway technique with either 0.005 N or 0.01 N-HCl solution for the titration, depending on the concentration of glutamine. Enzymic blanks containing water instead of the extract, and substrate blanks containing KCN-phosphate solution instead of the enzyme suspension, were carried out simultaneously. The net ammonia content after both blanks have been subtracted represents the amount of glutamine in 1 ml. sample.

RESULTS

The amount of ammonia liberated under the influence of the kidney enzyme preparation, was proportional to the amount of glutamine added in the range from 0.1 to 3 μmoles glutamine per ml. (Fig. 1).

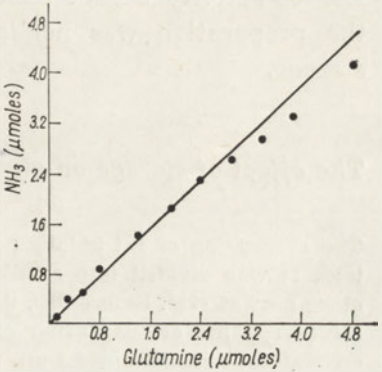

Fig. 1. The estimation of glutamine in standard solutions by the enzymic method. Each sample contained 50 μmoles phosphate; 25 μmoles tris, pH 7.7; 12 μmoles KCN, and an amount of enzyme preparation corresponding to 80 mg. of kidney tissue. Glutamine was added as indicated in the Figure. Final volume 1.3 ml. The incubation was carried out in the outer chamber of Conway microdiffusion dish at about 25° for 45 min.

The specificity of the enzyme preparation from rat kidney is demonstrated in Table 1. No ammonia, or only negligible amounts were liberated from adenine nucleotides, adenosine, and from the amino acids tested. The only exception was D,L-alanine from which about 40% of its ammonia content was liberated. As the deamination of L-alanine is

Table 1

The specificity of glutamine amidohydrolase preparation from rat kidney

Each sample contained 0.5 μmole substrate, 50 μmoles phosphate, 25 μmoles tris, pH 7.7, 12 μmoles KCN and suspension of the enzyme preparation corresponding to 80 mg. of tissue in the final volume 1.3 ml. Incubation was carried out in the outer chamber of Conway dish at 20 - 25° for 45 min. The reaction was stopped by the addition of 1 ml. saturated Na_2CO_3, which at the same time started ammonia microdiffusion

Substrate (0.5 μmole)	NH_3 liberated (μmoles)	Substrate (0.5 μmole)	NH_3 liberated (μmoles)
L-Glutamine	0.51	L-Serine	0.01
5′-AMP	0.00	D,L-Lysine	0.02
3′-AMP	0.00	D,L-Threonine	0.00
Adenosine	0.02	Glycine	0.00
L-Glutamic acid	0.00	L-Valine	0.00
L-Aspartic acid	0.01	L-Tyrosine	0.00
L-Asparagine	0.01	L-Leucine	0.00
D,L-Alanine	0.21	Taurine	0.00
L-Alanine	0.01	D-Glucosamine	0.00

negligible it is possible that only D-alanine, besides glutamine, was deaminated by our enzyme preparation.

The activity of glutamine amidohydrolase was fairly stable in the preparation stored at 1°, however, it decreased rapidly when the preparation was frozen and thawed (Table 2). When kept at 1°, the preparation retained its initial activity for about a fortnight, and then some loss of activity began. Usually the amount of endogenous ammonia in the preparation was negligible, however, it increased slightly during storage.

Table 2

The effect of storage on glutamine amidohydrolase activity in the enzyme preparation

The suspension of rat kidney particulate fraction was prepared as described in the text. It was divided into small portions, part of which was stored at 1° and the other part at -10°. Before use, the suspension was diluted twice with KCN-phosphate solution (7 ml. of suspension corresponded to 1 g. of tissue), and its activity was estimated by incubating 0.3 ml. with 10 μmoles glutamine under conditions described in Fig. 1, except that incubation time was 20 min.

Days of storage	Glutamine decomposed (μmole/20 min.)	
	Enzyme stored at 1°	Enzyme stored at -10°
0	2.59	2.59
5	2.24	0.35
8	2.15	0.34
12	2.28	0.39
17	1.01	
20	1.01	
23	1.13	
28	0.87	

The extraction procedure did not destroy the glutamine present in the tissue. This was proved by the recovery of the glutamine added to the deproteinized blood. A typical experiment of this series is shown in Table 3.

The determination of glutamine in the whole blood, without prior extraction or haemolysis, gave lower results; e.g. in two blood samples 0.49 and 0.42 μmoles glutamine per g. could be detected when incubated directly, and 0.84 and 1.04 μmoles per g. after extraction. This indicates that the extraction of the blood (or other tissues) forms an essential step in the procedure.

Reproducibility of the method was tested by performing ten estimations on the same human blood sample. The mean value obtained was 1.33 μmoles glutamine per g. with a standard deviation of 0.06. In Table 4

Table 3

The recovery of glutamine added to the blood

Glutamine was added to the blood deproteinized with trichloroacetic acid. Another portion of glutamine was diluted with water in the same proportion and glutamine was estimated in both samples as described in the text

Experiment	Glutamine (μmoles)	
	added	recovered
I	0.47	0.45
II	0.41	0.44
III	0.79	0.85

Table 4

Glutamine concentration in the blood of healthy human subjects fasted for 12 hr.

Initals	Glutamine (μmole/1 g. of blood)
J.P.	0.82
L.Ż.	0.80
M.Ż.	0.69
R.K.	0.88
W.M.	0.77
J.Pr.	0.88
W.Rz.	0.82
A.G.	0.52
J.U.	0.52
Z.T.	0.99
Average±S.D.	0.77±0.15

glutamine concentration in the blood of ten healthy human subjects aged 20 - 40 years fasted for about 12 hr., is shown. The concentration found is within the limits reported for blood plasma by other authors using different methods [e.g. 16]. Higher values were obtained when the blood had been taken after the ingestion of a meal.

DISCUSSION

The quantitative estimation of glutamine is of great interest for physiologists as this compound may constitute as much as 25% of total free amino acids in the blood and 60% in some other tissues [14]. Changes in glutamine concentration in the blood may occur in some pathological conditions [15, 5]. As already mentioned, few methods of glutamine determination have been described so far.

In our work advantage was taken of the fact that rat kidney glutamine amidohydrolase is present in the mitochondrial fraction [7] whereas AMP aminohydrolase is localized in the supernatant [17]. Using the KCN solution proposed by Archibald [1] to wash and suspend the mitochondrial fraction we were able to obtain a rather specific enzyme preparation. D-Alanine, which is deaminated by the preparation, does not occur in animal tissues and therefore could not interfere with the glutamine estimation. The kidneys of dog and cat could also be used for enzyme preparation. However, hog and ox kidney mitochondria, showing a high glutamine amidohydrolase activity, contain also adenosine aminohydrolase [11] and are not suitable for glutamine estimation in the presence of adenosine.

The method described is applicable to the blood and other animal tissues. Probably it could be also used for glutamine determination in plant material.

The authors appreciate the skilful technical assistance of Mrs. Regina Kaczorowska.

SUMMARY

1. A specific glutamine amidohydrolase preparation was obtained by suspending rat kidney mitochondrial fraction in KCN-phosphate solution.

2. By using this preparation, and the Conway technique for the estimation of ammonia, it is possible to determine even 0.1 μmole glutamine per ml. of blood or tissue extract.

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ENZYMATYCZNE OZNACZANIE GLUTAMINY PREPARATEM Z NERKI SZCZURA

Streszczenie

1. Przez zawieszenie frakcji mitochondrialnej nerek szczura w roztworze KCN zawierającym fosforany, otrzymano swoisty preparat glutaminazy.

2. Używając tego preparatu dla uwolnienia amoniaku i mikrodyfuzji Conwaya dla jego oznaczenia, można było oznaczyć 0,1 μmola lub więcej glutaminy w 1 ml ekstraktu krwi lub tkanki.

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INCORPORATION OF [2-^{14}C]URACIL INTO POLYNUCLEOTIDES IN HOMOGENATES OF WHEAT SEEDLINGS

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In the years preceding the discovery of polynucleotide phosphorylase, DNA polymerase and RNA polymerase, two different conceptions concerning the mechanism of the synthesis of polynucleotides were developed. According to one of them the nucleic acid molecule is formed by gradual binding of phosphates, carbohydrates and bases to a macromolecular acceptor. The other conception assumed the possibility of polymerization of activated mononucleotides (see Brown & Roll [3]).

It is now generally accepted that nucleosidediphosphates and nucleosidetriphosphates are the direct precursors of each nucleotide unit of the polynucleotide chain in animal tissues and in microorganisms as well as in higher plants [4, 2, 1, 8]. However, Reifer and co-workers [6, 7] in their studies on the anabolism of pyrimidines in higher plants *in vivo* made some observations which seem to indicate that mononucleotides may be not the only precursors of nucleic acids. First, no correlation was observed between the specific activities of both 5′-UMP and 5′-CMP and the corresponding 2′(3′)-mononucleotides obtained on hydrolysis of nucleic acids [6]. Then it was found that feeding of the plants with 0.05 mM solution of [2-^{14}C]uracil led to the incorporation of radioactivity into uridine and the polynucleotides, while 5′-UMP and 5′-CMP remained free of the radioactive carbon [7]. These observations suggested the possibility of direct incorporation of uracil or uridine into a fraction of polynucleotides, with the omission of the mononucleotide stage. In this paper, the corresponding results obtained *in vitro* on homogenates of plant material are reported.

MATERIALS AND METHODS

Homogenates. These were prepared in a cold-room at 6°; 20 g. samples of 5 days old wheat seedlings (Dańkowska 40 variety) were ground in a porcelain mortar with 20 ml. of ice-cold 0.2 M-saccharose solution containing 0.2 μmole of ATP per ml. The pulp was then passed through four layers of gauze and the liquid directly used in the preparation of

the incubation mixtures. Total protein content in the homogenate was 5 mg./ml.

Incubation. [2-^{14}C]Uracil (0.01 μmole in 0.5 ml. of 0.2 M-saccharose) with the specific activity of 20 μc/μmole was added to 2 ml. of the homogenate, made up to 3 ml. with 0.2 M-saccharose and incubated at 17°. The reaction was stopped by addition of 20 ml. of ice-cold 0.3 N-$HClO_4$. In the control sample, perchloric acid was added simultaneously with the radioactive uracil. After addition of perchloric acid the incubation mixtures were separated by centrifugation into two fractions: acid-soluble (supernatant) and acid-insoluble (precipitate).

Analytical procedures. The precipitate containing the polynucleotide was washed 10 times with 20 ml. portions of an ice-cold 0.1 mM-solution of non-radioactive uracil in 0.3 N-$HClO_4$, and hydrolysed in 1 N-HCl at 100° for 1 hr. After cooling, the hydrolysate was filtered and the solution either used for the determinations of total radioactivity of the polynucleotide fraction or subjected to further fractionation as described earlier [6].

The supernatant, i.e. the acid-soluble fraction, was heated for 10 min. on a boiling water bath in order to hydrolyse UDP and UTP into 5′-UMP, then cooled and the acid-soluble pyrimidine derivatives were separated according to the previously described procedure [6, 5].

Quantitative determinations and measurements of radioactivity were performed as before [6]. The particular pyrimidine derivatives were identified spectrophotometrically [10].

Chemicals. 2′(3′)-UMP was prepared from RNA according to the procedure described earlier [6]. Other reagents were obtained from commercial sources: [2-^{14}C]uracil, Radiochemical Centre, Amersham, England; ATP-Na_4 and ribose-5-phosphate (R-5-P), A. G. Fluka, Switzerland; glutathione, T. Schuchardt, München; potassium phosphates, E. Merck, Darmstadt; uridine, California Corp. for Biochemical Research; RNA, Nutritional Biochemicals Co. The solutions for preparing the incubation mixture were made up with double-distilled water.

RESULTS

The use of a saccharose solution containing ATP, a factor preventing heat denaturation of proteins [9], enabled to obtain a homogenate of wheat seedlings catalysing the incorporation of [2-^{14}C]uracil into the polynucleotides (Table 1). The homogenate prepared in this way showed no increase in activity on further addition of ATP, $MgCl_2$, R-5-P and glutathione. In homogenates prepared in 0.2 M-saccharose without ATP, [2-^{14}C]uracil was incorporated into the polynucleotides only in traces, the subsequent addition of ATP, $MgCl_2$, R-5-P or of all these compounds together to the incubation mixture remaining without effect (Table 1).

Table 1

Incorporation of [2-^{14}C]uracil into the polynucleotide fraction in the homogenates of wheat seedlings

Conditions: 2 ml. samples of homogenates were incubated for 1 hr. at 17° with 0.01 μmole of [2-^{14}C]uracil (20 μc/μmole). ATP, R-5-P, $MgCl_2$ and glutathione were added in amounts given in the Table. The incubation mixtures were made up to 3 ml. with 0.2 M-saccharose.

Mean values ± S.D. are given. In parentheses the number of experiments. The standard deviation was calculated from the equation: S.D. $= \sqrt{\frac{\Sigma (x-\bar{x})^2}{n-1}}$

Homogenate	Additions (μmoles)	Activity of polynucleotide fraction (counts/min.)
0.2 M-saccharose with 0.2 mM-ATP	None	30.3 ± 1.6 (5)
	ATP (0.4)	28.9 ± 2.3 (3)
	$MgCl_2$ (1.0)	30.7 ± 1.8 (3)
	R-5-P (0.4)	31.2 ± 1.3 (3)
	ATP (0.4) + $MgCl_2$ (1.0) + R-5-P (0.4)	30.1 ± 1.0 (3)
	Glutathione (2.0)	22.0 ± 1.9 (3)
0.2 M-saccharose	None	0.9 ± 0.5 (6)
	ATP (0.4)	1.0 ± 0.6 (6)
	$MgCl_2$ (1.0)	1.0 ± 0.4 (6)
	R-5-P (0.4)	0.7 ± 0.5 (6)
	ATP (0.4) + $MgCl_2$ (1.0) + R-5-P (0.4)	1.1 ± 0.8 (6)
	Glutathione (2.0)	0.3 ± 0.3 (6)

Table 2

Effect of pH on the incorporation of [2-^{14}C]uracil

Conditions: 2 ml. samples of homogenates in ATP-saccharose solution were incubated for 1 hr. at 17° with 0.01 μmole of [2-^{14}C]uracil (20 μc/μmole). The pH of the incubation mixture was adjusted by adding 40 μmoles of appropriate potassium phosphate buffer. The final volume of the incubation mixture was 3 ml. Mean values ± S.D. are given; in parentheses the number of experiments.

pH	Activity of polynucleotide fraction (counts/min.)
6.5 (without phosphate)	29.2±1.1 (5)
5.5	37.7±0.7 (3)
6.0	45.8±1.3 (3)
6.5	43.4±0.8 (5)
7.0	34.6±2.5 (3)
7.5	29.5±2.6 (3)

The addition of potassium phosphate to the incubation mixtures showed that its presence as well as changes of pH values influence essentially the intensity of uracil incorporation into the polynucleotides (Table 2). The highest radioactivity in this fraction was observed at pH 6. The total radioactivity of polynucleotides after 1 hr. of incubation amounted in this case to 0.25% of [2-^{14}C]uracil used.

In the subsequent experiments homogenates prepared in saccharose solution containing ATP were used, and their pH was adjusted before incubation to 6 with phosphate buffer.

The relation between the radioactivity of the polynucleotide fraction and the time of incubation is shown in Fig. 1. There was no incorporation

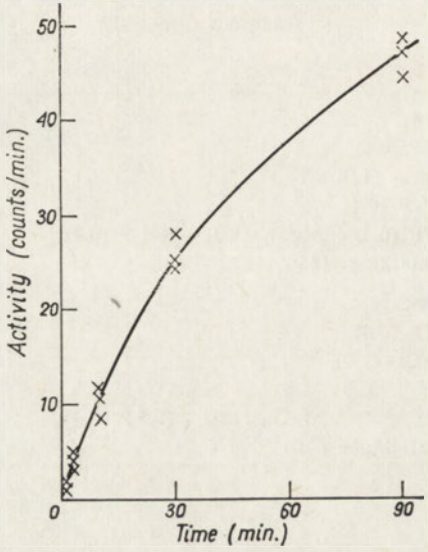

Fig. 1. Increase of activity of the polynucleotide fraction during incubation. Conditions: 2 ml. of homogenate in ATP-saccharose solution, 0.01 μmole of [2-^{14}C]uracil (20 μc/μmole), 40 μmoles potassium phosphate, 0.2 M-saccharose to 3 ml.; pH 6.0; temp. 17°. Results from three experiments.

of ^{14}C into the polynucleotides in the control sample. Already after 1 min. of incubation the activity appeared in the polynucleotide fraction and the linear increase of the activity was maintained for the first 30 min. Later the rate of uracil incorporation was distinctly lower.

The results of measurements of the content and activities of some pyrimidine derivatives in the homogenates incubated with [2-^{14}C]uracil during various periods, are presented in Table 3. In uridine and uridine mononucleotides only insignificant amounts of ^{14}C were found, irrespective of the incubation time. Considerable activity was, however, found after 90 min. of incubation in the hydrolysis products of polynucleotides

Table 3

Content and total activity of some pyrimidine derivatives in homogenates of wheat seedlings incubated with [2-^{14}C]uracil

Conditions: 2 ml. samples of homogenates in ATP-saccharose solution were incubated with 0,01 μmole of [2-^{14}C]uracil (20 μc/μmole), 40 μmoles of potassium phosphate, and 0.2 M-saccharose up to 3 ml.; pH 6.0; temp. 17°. Time of incubation as indicated in the Table. Mean values from three experiments ± S.D. are given

Pyrimidine compounds	Time of incubation (min.)							
	0 (Control)		1		10		90	
	μmole	counts/ min.	μmole	counts/ min.	μmole	counts/ min.	μmole	counts/ min.
Acid-soluble fraction								
Uracil	0.06	17020±30	0.06	17040±30	0.08	17010±30	0.07	16880±30
Uridine	0.13	1.0±0.3	0.12	1.2±0.9	0.12	1.4±0.8	0.14	0.9±0.2
5′-UMP+ +UDP+UTP	0.10	0.8±0.4	0.11	1.3±0.5	0.13	1.3±0.4	0.13	1.0±0.5
Acid-insoluble fraction								
2′(3′)-UMP	0.46	0.2±0.3	0.46	1.3±0.2	0.47	5.6±1.3	0.43	49.8±2.4
Uridine	0.05	0.3±0.3	0.04	1.2±1.6	0.05	2.6±0.9	0.05	15.4±1.3

namely in 2′(3′)-UMP and uridine. Presumably the homogenates did not catalyse the degradation of [2-^{14}C]uracil, for its loss during incubation was very slight. The content of the particular pyrimidine derivatives did not undergo any considerable changes in the course of the incubation.

DISCUSSION

The presented data show that in the homogenates of wheat seedlings incubated with [2-^{14}C]uracil, considerable activity appears in the hydrolysis products of the acid-insoluble fraction. The lack of incorporation of ^{14}C in the control, as well as in homogenates prepared without ATP indicates the enzymic character of this process. The observed dependence of uracil incorporation on the pH value and on the incubation time is also typical for enzymic reactions.

The presence of radioactive 2′(3′)-UMP and uridine in the products of hydrolysis of the acid-insoluble fraction suggests that the observed incorporation of [2-^{14}C]uracil is connected with the biosynthesis of polynucleotides. Although the information so far obtained is inadequate to elucidate the mechanism of this biosynthetic process, yet it is possible to draw some tentative conclusions. First, the relatively high radioactivity of uridine formed on hydrolysis of polynucleotides in comparison

with the activity of 2′(3′)-UMP indicates that a considerable part of the incorporated uracil results from the terminal addition. Secondly, the attention is called to the lack of ^{14}C in the uracil derivatives of the acid-soluble fraction, namely in uridine and uridine mononucleotides. These observations would indicate that uracil can be incorporated directly into the respective fraction of polynucleotides with the omission of uridine and of mononucleotides. In other words, the incorporation of radioactivity into the nucleic acids occurring under the described experimental conditions seems to result from direct binding of [2-^{14}C]uracil by a polynucleotide acceptor rather than from the polymerization of mononucleotides.

The possibility of anabolic reactions of uracil in plants was recently suggested on the basis of experiments *in vivo* [7]. The results obtained *in vitro* support this finding.

The author wishes to express his gratitude to Professor I. Reifer for valuable suggestions during the course of this work.

SUMMARY

Homogenates of wheat seedlings were incubated with [2-^{14}C]uracil. A high rate of incorporation of ^{14}C into the acid-insoluble fraction was observed. On hydrolysis, two radioactive products were identified, namely 2′(3′)-UMP and uridine. In the acid-soluble fraction no products of metabolism of [2-^{14}C]uracil were found. These results suggest a possible direct path of incorporation of uracil into polynucleotides, with the omission of the mononucleotide stage.

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WŁĄCZANIE [2-^{14}C]URACYLU DO POLINUKLEOTYDÓW W HOMOGENATACH KIEŁKÓW PSZENICY

Streszczenie

Homogenaty kiełków pszenicy inkubowano z [2-^{14}C]uracylem. Stwierdzono szybkie włączanie ^{14}C do frakcji kwasonierozpuszczalnej. Po hydrolizie otrzymano dwa radioaktywne związki: 2′(3′)-UMP i urydynę. We frakcji kwasorozpuszczalnej nie znaleziono żadnych produktów przemian [2-^{14}C]uracylu. Wyniki sugerują możliwość bezpośredniego włączania uracylu do polinukleotydów a nie poprzez mononukleotydy.

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SEASONAL CHANGES IN THE EXCRETION OF NITROGEN WASTES IN *HELIX POMATIA*

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In an earlier paper [1] we have stated an accumulation of uric acid, xanthine and guanine in the nephridia of hibernating *Helix pomatia*. In spring, when the snails resumed their activity, a great part of these purine compounds was eliminated but a considerable proportion remained stored in nephridium. During this investigation extending till July almost the whole of nitrogen substances in nephridium and nephridial excreta consisted of the same purines only with changes in their mutual proportion. In contrast with the accepted view that *Helix pomatia* is uricotelic during hibernation but ureotelic during the feeding period, our conclusion was that *H. pomatia* is uricotelic throughout the whole year.

The present investigation was carried out from August till March completing the former observation so as to cover the whole year cycle.

Fully grown snails were collected and kept under the same conditions as described previously [1]. The first series of experiments was made in September and the beginning of October when the snails were feeding very actively. During the next period, from October 15th to November 15th, the feeding was quite irregular and in many specimens the closing of shell by epiphragma was observed. Some of them, however, resumed their activity and feeding. Therefore in this series our material was inhomogeneous. Beginning with the second half of November all through the winter till March the snails remained in shells closed with solid epiphragma.

The methods applied were the same as in [1]. The total nitrogen was estimated in isolated nephridia and in nephridial excreta. From chromatograms the spots of uric acid, guanine, xanthine and of an unidentified substance were eluted, and the nitrogen content of the three last compounds was estimated. The uric acid was estimated colorimetrically and its nitrogen content was calculated. Table 1 presents the results together with those from the earlier paper. The nitrogen of purine compounds is also expressed as percentage of total nitrogen. In Table 1 a the same results are expressed in μmoles and the difference for each

Table 1

The nitrogen compounds in nephridia and nephridial excreta of the snail Helix pomatia

The values are averages from determinations made on 3 - 4 snails. The results are expressed as mg. N per nephridium or per 100 mg. of excreta, and as percentages of total N. The figures for the periods marked with an asterisk are taken from the previous paper [1]. For November, December and February, the results for snails in which guanine was present are included in the set A, and for those without guanine, in the set B

Material	Period (months)		Total N	Nitrogen of										Nitrogen accounted for	
				uric acid		xanthine		guanine		total purines		unknown compound			
			(mg.)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)
Nephridium															
during hibernation	XI - XII	A	18.4	8.8	46.2	6.6	37.3	2.9	16.2	18.3	99.3	0.0	0.0	18.3	99.3
		B	23.6	12.6	52.7	10.0	42.7	0.0	0.0	22.6	95.7	0.0	0.0	22.6	95.7
	I		32.9	17.4	59.0	13.3	40.4	0.0	0.0	30.7	93.3	0.0	0.0	30.7	93.3
	II	A	39.0	19.7	50.5	11.3	27.9	4.7	12.0	35.7	95.5	0.0	0.0	35.7	95.5
		B	32.1	21.8	67.7	8.1	24.6	0.0	0.0	29.9	92.3	0.0	0.0	29.9	92.3
	end of II - III *		47.1	31.3	66.4	6.0	12.7	5.6	11.8	42.9	91.0	0.0	0.0	42.9	91.0
during the feeding period	IV - 15.VII *		21.3	7.5	35.2	7.2	33.9	4.2	19.7	18.9	88.7	1.9	8.6	20.8	97.6
	IX - X		13.1	3.4	25.9	6.2	47.1	2.4	19.1	12.0	93.1	0.7	5.1	12.7	97.8
	X - XI	A	19.1	9.2	48.1	7.3	37.4	2.4	12.8	18.9	98.3	0.3	1.3	19.2	99.6
		B	18.2	6.8	37.5	10.6	58.3	0.0	0.0	17.4	95.8	0.0	0.0	17.4	95.8
Excreta															
first after hibernation		*	33.6	21.7	64.6	12.6	37.5	0.0	0.0	34.3	102.1	0.0	0.0	34.3	102.1
during the feeding period	IV - 15.VII		33.0	14.2	43.0	9.9	30.0	6.0	18.2	30.2	91.5	1.5	4.5	31.7	96.1
	IX - X		31.4	9.9	31.5	15.9	50.9	3.9	12.6	29.7	94.2	1.0	3.3	30.7	97.5

Table 1a

Purines in nephridia and nephridial excreta of the snail Helix pomatia

The values represent the amount of purines in μmoles per 1 nephridium or per 100 mg. of excreta. The average for the whole year and the differences between the found values and the year's average (d.y.a.) are also given. The figures for the periods marked with an asterisk are taken from the previous paper [1]. For November, December and February, the results for snails in which guanine was present are included in the set A, and for those without guanine, in the set B

Material	Period (months)	Uric acid		Xanthine		Guanine		Sum for xanthine and guanine	
		(μmoles)	(d.y.a.)	(μmoles)	(d.y.a.)	(μmoles)	(d.y.a.)	(μmoles)	(d.y.a.)
Nephridium during hibernation	XI - XII A	157	—87.6	119	—35.3	44	+11.8	320	—111.1
	B	162	—82.6	178	+23.7	0	—32.2	340	—91.1
	I	347	+102.4	237	+82.7	0	—32.2	584	+152.9
	II A	352	+107.4	200	+45.7	67	+34.8	619	+187.9
	B	389	+144.4	145	—9.3	0	—32.2	534	+102.9
	end of II - III *	559	+314.4	107	—47.3	80	+47.8	746	+314.9
during the feeding period	IV - 15.VII *	135	—109.6	127	—27.3	62	+29.8	324	—107.1
	IX - X	60	—184.6	110	—44.3	35	+2.8	205	—226.1
	X - XI A	163	—81.6	131	—23.3	34	+1.8	328	—103.1
	B	122	—122.6	189	+34.7	0	—32.2	311	—120.1
	Year's average	244.6		154.3		32.2		431.1	
Excreta first after hibernation	*	389		226		0		615	
during the feeding period	IV - 15.VII *	261		176		86		523	
	IX - X	177		285		56		518	

period from the average for the whole year is calculated. The results for guanine in November and December, and again in February were highly variable within the series of experiments. In some snails guanine was not to be detected at all, in many others the amount of this base was rather remarkable. We were inclined to refer these findings to the already mentioned differences in behaviour of snails in the period at the onset and towards the end of hibernation. Therefore in the Tables the results were pooled into two sets, A and B, B representing the experiments in which no guanine could be detected. The seasonal fluctuations

of the content of purine substances in nephridia are graphically presented in Fig. 1 giving the amount in μmoles throughout the year.

The total nitrogen in nephridium varied markedly throughout the year exhibiting a minimum towards the end of the feeding period. Soon afterwards the epiphragma was formed and the excreta could not be eliminated. This is reflected by a rise of nitrogen compounds in nephri-

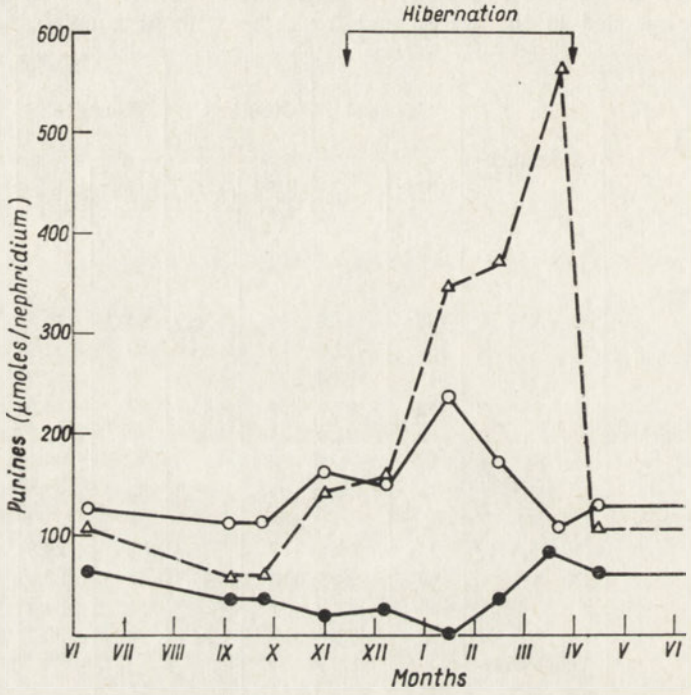

Fig. 1. Changes in the content of purines in nephridia of *H. pomatia* throughout the year. (△), Uric acid; (○), xanthine; (●), guanine. The results for November, December and February are averages of the results for the sets A and B of Table 1a

dia proceeding gradually till the end of hibernation, the value for the maximum being three times that for the minimum. After awakening, the snails deposited the excreta, and the nitrogen content in nephridia fell down rapidly to a half of the maximum value. Then the decrease proceeded slowly during the feeding period reaching the minimum in autumn.

The composition of nephridial excreta remained unchanged throughout the whole feeding period. In nephridia during the whole year over 90% of total nitrogen consisted of purine derivatives. During hibernation uric acid formed the greatest part of total N. The amount of uric acid increased tenfold over this period, and towards the end of hibernation almost 65% of total N consisted of this compound.

The rapid augmentation of uric acid towards the end of hibernation is clearly expressed in Fig. 1. The excreta eliminated just after awakening contained predominantly uric acid, and that resulted in a rapid decrease of this compound in nephridia leaving only one third of the maximum value. During the feeding period the level of uric acid in nephridia fluctuated but slightly, decreasing slowly during autumn. Just before the onset of hibernation the uric acid was at its minimum contributing as little as 25% to total nitrogen. Reciprocally, in the excreta deposited just after awakening the uric acid constituted as much as 60% of total N, and during the feeding period only 30%.

The percent contribution of xanthine to total N was at its maximum just before the onset of hibernation when it constituted 47 - 58% of total N. In early stages of hibernation the absolute content of xanthine rose until January when it reached its maximum but the percent contribution fell at the same time being overtaken by the more rapid rise of uric acid. At its maximum, the absolute content of xanthine was twice that at the onset of hibernation. In February and March the level of xanthine in nephridia was falling, reaching its minimum just before the snail resumed in spring its activity. At this moment the nitrogen of xanthine furnished only 12% of total N which is the relative minimum throughout the year. During the feeding period the level of xanthine fluctuated but slightly almost parallelly to changes in uric acid.

In excreta, the xanthine nitrogen contributed throughout the feeding period 30% of the total N. This value rose markedly towards the end of the active period reaching 51% of total N. After snails' awakening, the xanthine made 37% of the excreted nitrogen.

Guanine is the least soluble of the three purine compounds. Its content diminished gradually during the feeding period and during the first half of hibernation. In January this base was absent from the nephridia of all snails examined. In November, December and February the same was true for many of the specimens examined but at the same time in other specimens the content of guanine was quite conspicuous, sometimes reaching the same values as during the feeding period. We have already discussed this bifurcation when speaking about the results presented in Table 1.

The maximum of guanine was to be found in March simultaneously with the maximum for uric acid and the minimum for xanthine content. After snails' awakening, a gradual fall of guanine content began and was maintained through the feeding period. Guanine was not observed in the excreta of snails just after awakening. During the feeding period this base made about 18% in spring and summer, and about 13% in autumn, of the total N of the excreta.

In Fig. 1 the curves for xanthine and guanine are somewhat reciprocal so that in nephridium the sum of both would vary but slightly throughout

the year. In consequence, the great variations in the storage of nitrogen throughout the year are due mainly to storage or elimination of uric acid.

The unidentified compound absorbing in UV accumulated in nephridia only during the feeding period, being at its maximum early in spring (almost 9% of total N). It was absent from excreta deposited after awakening and made 4.5% and 3.3% of the total N of excreta during summer and autumn, respectively. It seems to be some constituent of the ingested food.

Our present investigation substantiated the suggestion that *Helix pomatia* is uricotelic throughout the year.

SUMMARY

The nitrogen compounds in nephridia and nephridial excreta of *Helix pomatia* were examined throughout the year. It was found that in all seasons *H. pomatia* is uricotelic, as over 90% of N-wastes consists of purine compounds. In spring, the nephridia contain almost equal amounts of xanthine and uric acid, in autumn xanthine prevails both in nephridia and in excreta. During the first half of hibernation there is a parallel rise in xanthine and uric acid, in the second half, uric acid is still rising but xanthine is diminishing being substituted by guanine.

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ROCZNY CYKL WYDALANIA ZWIĄZKÓW AZOTOWYCH U *HELIX POMATIA*

Streszczenie

Przebadano wydalanie związków azotowych przez *Helix pomatia* w ciągu roku. Stwierdzono, że przez cały rok ślimak winniczek jest urikoteliczny. Ponad 90% wydalin azotowych stanowią puryny. Nefridium żerujących ślimaków zawiera na wiosnę prawie równe części ksantyny i kwasu moczowego, na jesieni przeważa ksantyna zarówno w nefridium jak i w jego wydalinach. W pierwszej połowie okresu hibernacji wzrost kwasu moczowego i jego prekursora, ksantyny, jest prawie równoległy, natomiast w drugiej połowie ilość ksantyny zmniejsza się i nefridium zawiera głównie słabo rozpuszczalne w wodzie puryny, guaninę i kwas moczowy.

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SIALIC ACID AND OTHER PROTEIN-BOUND CARBOHYDRATES IN EHRLICH MOUSE ASCITES TUMOUR FLUID*

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Ehrlich ascites tumour seems to be a suitable object for studying the biosynthesis of sialic acid *in vitro*. Wallach & Eylar [12] in 1961 found about 11 mg. sialic acid per 100 ml. of packed ascites tumour cells. In the present work about 80 mg. sialic acid per 100 ml. of ascites fluid was found.

In various animal species and in various tissues different sialic acid derivatives are present, e.g. *N*-acetylneuraminic acid in human serum, *N*-glycollylneuraminic acid in hog submaxillary gland, and a mixture of various sialic acids comprising a great amount of *N,O*-diacetylneuraminic acid in bovine submaxillary gland [5]. The present paper describes an attempt to determine which derivatives of neuraminic acid are present in ascites fluid. Also the amount of protein-bound carbohydrates was assayed in ascites fluid and in blood sera of normal and of tumour-bearing mice.

METHODS

Ehrlich ascites tumour cells were obtained from the Department of Pathological Anatomy of the Medical School in Gdańsk. Ten days after intraperitoneal inoculation of white mice with Ehrlich ascites tumour cells, the ascites fluid was withdrawn from the peritoneal cavity. The fluid from individual mice was centrifuged; the supernatants were combined, and used for further experiments.

The serum was obtained from pooled blood of 15 decapitated normal mice and from pooled blood of 15 tumour-bearing mice decapitated 10 days after the inoculation.

Sialic acid was determined by the method of Svennerholm [11] using as a standard *N*-glycollylneuraminic acid kindly given by Professor G. Blix. The sialic acid-protein complex was hydrolysed by the method of Martensson *et al.* [9]. The protein was precipitated with ethanol from 100 ml. of cell-free ascites fluid and hydrolysed twice with

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0.03 N-sulphuric acid for 1 hr. at 80°. From the combined hydrolysates sulphuric acid was removed with barium hydroxide and the clear solution was freeze-dried. The obtained material was dissolved in 3 ml. of water and placed on several sheets (15 × 45 cm.) of Whatman no. 1 paper. The chromatography was carried out in *n*-butanol - pyridine - water (6:4:3, by vol.) with the descending technique [2]. From the central part of each sheet, a strip 1 cm. wide, parallel to the direction of solvent migration was cut out, the spots were localized with orcinol in trichloroacetic acid [3], and compared with the spot of standard *N*-glycollylneuraminic acid. The areas corresponding to the most intensive spot were cut out from the non-stained chromatograms and eluted with water; the combined eluates were filtered through a hard, washed with water, filter paper, and freeze-dried. In this material dissolved in 10 ml. of water, the sialic acid [11], *O*-acyl [8] and *N*-glycollyl [6] residues were determined.

In the protein precipitated with ethanol, hexose was determined with orcinol [13], hexosamine by the Elson-Morgan method after Blix [1] and fucose with the cysteine reagent [13]. The amount of protein was determined by the biuret method [7].

RESULTS

The protein-bound sialic acids present in ascites fluid were separated on chromatography into 3 spots (Fig. 1). The first spot with a mobility of standard *N*-glycollylneuraminic acid constituted the main component, whereas two other spots formed a much smaller fraction. The compound

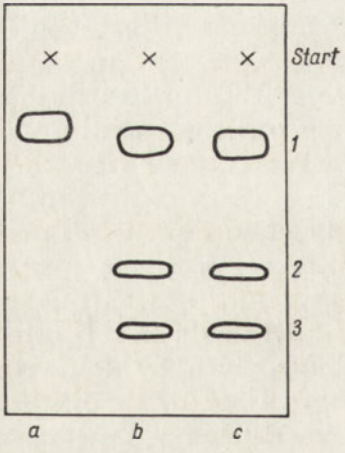

Fig. 1. Paper chromatography of sialic acid separated from Ehrlich ascites fluid protein. Descending technique in *n*-butanol - pyridine - water (6:4:3, by vol.). (a), Standard *N*-glycollylneuraminic acid; (b), mixture of sialic acids from ascites with *N*-glycollylneuraminic acid; (c), sialic acids from ascites.

corresponding to the first spot was eluted from chromatograms with water. In the eluate, per 1 mole sialic acid nearly 1 mole of *N*-glycollyl residues was found, and no *O*-acyl residues could be detected (Table 1).

Table 1

N-Glycollyl and O-acyl residues of sialic acid isolated from Ehrlich ascites fluid protein

Sialic acid eluted from the chromatogram (spot 1 in Fig. 1) was tested for *N*-glycollyl and *O*-acyl residues

Expt. no.	*N*-Glycollyl residue (mole per mole sialic acid)	*O*-Acyl residue (mole per mole sialic acid)
1	0.81	0.0
2	0.73	0.0
3	0.90	0.0
4	0.81	0.0
Mean value ± S.D.	0.81 ± 0.11	0.0

The above data indicate that in Ehrlich ascites tumour fluid the main protein-bound neuraminic acid derivative is *N*-glycollylneuraminic acid.

In Table 2 are presented the results of determinations of protein-bound carbohydrate components, and of sialic acid, in mouse ascites fluid. It should be noted that the standard deviation for the determinations of carbohydrate per gram of protein was very small. The average amount of sialic acid, 85 mg. per 100 ml. ascites fluid, was much higher than that in tumour cells which was found by Wallach & Eylar [12] to be 11 mg. per 100 ml. cells.

In Table 3 the amounts of individual carbohydrate components in ascites fluid are compared with those for the serum of normal and

Table 2

Protein-bound carbohydrate components in Ehrlich ascites tumour fluid

Expt. no.	Protein (g./100ml.)	Hexose (mg./100 ml.)	Hexosamine (mg./100 ml.)	Sialic acid (mg./100 ml.)	Hexose (mg./g. protein)	Hexosamine (mg./g. protein)	Sialic acid (mg./g. protein)
1	2.5	82	71	81	33	28	32
2	2.4	79	53	82	32	22	33
3	2.7	97	72	103	39	26	38
4	2.7	89	80	93	33	29	34
5	2.7	84	63	80	31	23	30
6	2.3	82	60	80	35	26	35
7	2.4	84	58	80	35	26	35
Mean values ± S. D.	2.5±0.06	85.2±2.2	65.2±3.5	85.5±3.4	34.0±1.0	25.7±0.89	33.8±0.9

Table 3

Protein-bound carbohydrate components in sera of normal and tumour-bearing mice and in Ehrlich ascites fluid

Material	Protein	Hexose	Hexosamine	Sialic acid	Fucose
	(g./100 ml.)	(mg./100 ml.)			
Serum of normal mouse	7.0	114	95	130	4.8
Serum of tumour-bearing mouse	7.0	160	150	120	4.4
Ascites fluid	2.5	85	65	85	—
		(mg./g. protein)			
Serum of normal mouse		16.3	14	18	0.7
Serum of tumour-bearing mouse		23	21	17	0.6
Ascites fluid		34	26	34	—

tumour-bearing mice. In the protein of the tumour-bearing mouse serum, the content of hexose and hexosamine was higher than in the serum of normal mice, whereas the amounts of sialic acid and fucose were not changed. In the protein of ascites fluid about twice as much carbohydrates was found as in the serum of normal mice. The amount of sialic acid per 1 g. protein was twice as high in ascites fluid as in the serum of normal or tumour-bearing mice.

The amounts of fucose in ascites fluid were much smaller than in the serum and could not be determined with the cysteine reagent.

DISCUSSION

In mouse ascites fluid, three sialic acid derivatives were found by paper chromatography. The main one is probably *N*-glycollylneuraminic acid. Although this compound was not obtained in pure crystallized form, the R_F value, and the analysis of *N*-glycollyl and *O*-acyl groups strongly support this suggestion. The two other sialic acids could be *O*-acetyl derivatives (spots 2 and 3 in Fig. 1). They were not identified but there is a marked similarity between our chromatogram and that of Blix [2] obtained with *O*-acetyl derivatives.

Higher content of sialic acid in ascites fluid protein than in the protein of normal and tumour-bearing mouse serum, can be explained by the assumption that the proteins containing acid polysaccharides are produced by tumour cells or by the peritoneal membrane, and cannot pass into the blood. Other glycoproteins synthesized by tumour cells or by the peritoneal membrane may penetrate into the blood causing the increase of hexose and hexosamine contents in the serum of tumour-bearing mice.

On the other hand, it cannot be excluded that in tumour-bearing mouse the increase of protein-bound hexose and hexosamine first occurs in the serum, from which these compounds would pass to the peritoneal cavity. The greater amount of sialic acid in the ascites fluid than in the serum could be accounted for by active accumulation against the concentration gradient.

The high rate of protein synthesis in Ehrlich ascites tumour cells was demonstrated by Le Page [10]. He found that the incorporation of [2-^{14}C]glycine by these cells was 50 times greater than by mouse liver cells. Christensen & Riggs [4] showed *in vitro* an assimilation of amino acids from the medium. Glycine gradients of 60 mM per liter were observed between the cells and the suspending fluids.

SUMMARY

N-Glycollylneuraminic acid was found to be the main component of the sialic acids in the protein of Ehrlich mouse ascites fluid. In the serum of the tumour-bearing mouse an increase of protein-bound hexose and hexosamine in relation to normal mouse serum was observed; however, no differences in sialic acid content in the sera were found.

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KWAS SJALOWY ORAZ INNE KOMPONENTY WĘGLOWODANOWE W PŁYNIE ASCITES EHRLICHA U MYSZY

Streszczenie

Stwierdzono, że kwas *N*-glikoliloneuraminowy stanowi główną komponentę kwasów sjalowych białek otrzewnowego płynu nowotworowego myszek z guzem Ehrlicha. Porównano zawartość składników węglowodanowych białek płynu otrzewnowego i surowicy myszek zdrowych i nowotworowych. Zawartość heksozy i heksozaminy w surowicy myszek nowotworowych była wyższa około pół raza, a w płynie otrzewnowym około dwa razy w przeliczeniu na 1 g białka w porównaniu z ich zawartością w surowicy myszek zdrowych. Poziom kwasu sjalowego w przeliczeniu na 1 g białka w surowicy myszek zdrowych i nowotworowych był jednakowy, lecz dwukrotnie niższy niż w płynie otrzewnowym.

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RECENZJE KSIĄŻEK

Christopher Carruthers: BIOCHEMISTRY OF SKIN IN HEALTH AND DISEASE. Charles C. Thomas Publ., Springfield, Illinois, U.S.A. 1962. Str. 263, rys. 41, tabl. 130.

Omawiana książka jest kolejną pozycją serii "American Lectures in Living Chemistry" pod redakcją I. M. Kugelmassa, który ją poprzedził wstępem. Książka podzielona jest na 9 rozdziałów. W pierwszym z nich Autor podaje, głównie na podstawie prac C. C. Selby (1955), G. F. Odlanda (1958) oraz M. S. C. Birbecka i E. H. Mercera (1957), szczegóły ultrastruktury skóry ujawnione przez mikroskopię elektronową. W drugim rozdziale Autor omawia krótko białka skóry oraz proces keratynizacji, przyjmując teorię Flescha, wedle której keratynizacja przebiega w dwóch etapach: w pierwszym tworzy się włóknisty białkowy prekursor zawierający najwyżej ślady siarki, w drugim etapie ten prekursor łączy się z białkami bogatymi w siarkę lub produktami rozpadu takich białek wytwarzając keratynę. Trzeci rozdział poświęca Autor pigmentacji, omawiając melanogenezę i wpływ promieni pozafiołkowych. W czwartym rozdziale zbiera dane dotyczące rodzaju i zawartości substancji tłuszczowych. Rozdział piąty poświęcony jest enzymom występującym w skórze. W rozdziale szóstym omówiono skórę jako magazyn wody oraz chlorku i innych składników mineralnych, oraz przedstawiono dane dotyczące pH i potu. W rozdziale siódmym Autor omawia tkankę łączną, poświęcając osobny ustęp zagadnieniu gojenia ran. Rozdział ósmy zajmuje się włosami. Ostatni, dziewiąty, rozdział poświęcony jest chorobom skóry. Autor omawia najpierw zmiany zachodzące przy łuszczycy, następnie dyskutuje ogólnie zaburzenia metabolizmu w różnych chorobach skórnych, a dalej zmiany zachodzące w składzie surowicy krwi w poszczególnych chorobach.

Każdy rozdział kończy się zestawieniem literatury (do 1961 r.) zawierającym kilkadziesiąt pozycji; stanowią one cenne źródło informacji. Rozdziały o ultrastrukturze, tłuszczach, enzymach, o wodzie itd. oraz rozdział o chorobach skóry dotyczą głównie skóry ludzkiej, natomiast cztery pozostałe rozdziały opierają się na znacznie szerszej podstawie i częściowo na pracach eksperymentalnych wykonanych na zwierzętach.

Wkład Autora w opracowanie i przedstawienie danych zebranych z piśmiennictwa nie jest równomierny. Recenzent z przyjemnością przeczytał doskonale opracowany i uporządkowany rozdział o melanogenezie, natomiast rozdział o enzymach skóry, którymi Autor sam eksperymentalnie się zajmuje, zredagowany jest znacznie słabiej. W sumie książkę czyta się z przyjemnością; stanowi ona cenny zbiór informacji dla dermatologów i biologów, którzy badają procesy biochemiczne i biofizyczne związane ze skórą. Z zainteresowaniem przeczyta tę książkę każdy biolog zyskując właściwy pogląd na ten organ, który w podświadomości wielu biologów rysuje się jeszcze jako bierna powłoka odgraniczająca ustrój od świata zewnętrznego. Do tych ostatnich czytelników skierowana jest przedmowa redaktora serii monografii. Przypomina on, że każdy cm^2 skóry zawiera 3 000 000 komórek, 15 gruczołów łojowych, 10 włosków, około 1 metra bieżącego naczyń krwionośnych, 100 gruczołów potowych, 300 receptorów czuciowych z 4 metrami nerwów, w tym 25 receptorów reagujących na ciśnienie mechaniczne, 200 generujących uczucie bólu, 12 wrażliwych na ciepło, 2 na zimno. Nawet najbardziej zewnętrzna warstewka grubości 0.03 mm, którą kiedyś uważano za martwą, wykazuje fizyko-

chemiczne układy współdziałające w mechanizmach utrzymania temperatury ciała, pH powierzchni, obrony przed bakteriami i grzybkami, w transporcie wody i in. Skórę można więc uważać za złożony gruczoł wydzielający keratynę, melaninę, sebum, pot, heparynę i histaminę. Zasługuje ona niewątpliwie na większe zainteresowanie biologów.

Józef Heller

Hess B.: ENZYME IM BLUTPLASMA. (Biochemie und Klinik — Monographien in zwangloser Folge. Herausgeber: G. Weitzel, Tübingen; N. Zöllner, München). Georg Thieme Verlag. Stuttgart. 1962: 142 str., DM 29,90.

Zwięzłe monograficzne opracowanie doc. dr Hessa pt. Enzymy w Osoczu Krwi należy do zamierzonej serii wydawnictw pod ogólnym tytułem Biochemia i Klinika. Seria ta, według jej wydawców, ma na celu krytyczne przedstawienie tych działów biochemii, które mają znaczenie dla medycyny wewnętrznej. Książka została podzielona na dwie części, biochemiczną i kliniczną. Pierwsza część, stanowiąca prawie połowę objętości, zawiera wyczerpujący opis rodzajów i własności enzymów krwi, a także podaje nowoczesne zapatrywania na pochodzenie enzymów krwi, ich syntezę i eliminację względnie rozpad. Przy opisie enzymów autor powołuje się na terminologię i klasyfikację zaakceptowaną przez Międzynarodową Unię Biochemiczną, mimo to używa w wielu przypadkach dawnych nazw, chociaż zostały one zdyskwalifikowane (np. DPN zamiast NAD, enolaza zamiast hydrataza fosfopirogronianowa, transaminaza glutaminowo-szczawiowooctowa zamiast aminotransferaza asparaginianowa itd.). Chociaż dotychczasowe nazwy nie mogą być jeszcze zupełnie pominięte, nowe jednak powinny być stosowane coraz szerzej. Zagadnienie izoenzymów zostało przez autora obszernie i krytycznie przedstawione i to zarówno z punktu widzenia teoretycznego jak i praktycznego. Opierając się głównie na wynikach szkoły Büchera autor przedstawił nowoczesne poglądy na znaczenie wzajemnego ilościowego stosunku enzymów w różnych tkankach, uwzględnił przy tym znaczenie filogenezy dla „zestawów" enzymów. Biochemiczna część zawiera na końcu opis stanu dzisiejszej wiedzy na temat korelacji między aktywnościami charakterystycznymi dla poszczególnych rodzajów enzymów w surowicy i stopniem zmian patologicznych. Część kliniczna książki została usystematyzowana według podziału klinicznego, przy czym najwięcej miejsca poświęcono chorobom serca, wątroby i trzustki. Omówiono przy tym oddzielnie patogenezę i diagnostykę schorzeń tych organów. Sposób przedstawienia umożliwia czytelnikowi ocenę stopnia ważności wyników oznaczania aktywności enzymatycznych poszczególnych enzymów. Autor porównał także wyniki oznaczeń aktywności enzymowej z wynikami różnych prób diagnostycznych. Z innych chorób poświęcono więcej miejsca sprawom nowotworowym. Książkę zamyka rozdział, w którym podano jasno i krótko podstawowe definicje enzymologii ogólnej. Dla jednostki enzymatycznej przyjęto zalecenia Komisji Enzymowej Międzynarodowej Unii Biochemicznej. Cenna jest tabela umożliwiająca przeliczanie dotychczasowych różnych jednostek na jednolity sposób przyjęty na terenie międzynarodowym. Literatura przedmiotu obejmuje prawie sześćset pozycji. Szkoda, że nie zastosowano przy tym klucza alfabetycznego.

Książkę można polecić wszystkim, którzy interesują się zagadnieniami enzymologii klinicznej.

Tadeusz Korzybski