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A STUDY ON THE OXIDO-REDUCTIVE PROPERTIES OF CAERULOPLASMIN BY OSCILLOGRAPHIC POLAROGRAPHY

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1. Electrode processes of caeruloplasmin were observed by the alternating current oscillographic method, using a P-576 Křižik Polaroscope fitted with a dropping mercury electrode. 2. In 0.2 M-acetate buffer, pH 5.6, at 25° the presence of a reversible oxidation-reduction system in caeruloplasmin, with a Q value of 0.4, was demonstrated. 3. The activation by Fe^{2+} ion of caeruloplasmin oxido-reductive processes was confirmed for some biologically active compounds.

Attempts at elucidating the action of caeruloplasmin, a blue copper glycoprotein of blood plasma, toward *p*-phenylenediamine (Peisach & Levine, 1963), *N,N*-dimethyl-*p*-phenylenediamine and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (Curzon, 1967) have been recently reported. The activating effect of Fe^{2+} ion on the enzymic activity of caeruloplasmin was demonstrated by Curzon (1960). So far the effect of Fe^{2+} on oxidation by caeruloplasmin of physiologically active compounds: serotonin, adrenaline and noradrenaline, has not been thoroughly studied.

Several methods have been developed for studying the course of very rapid chemical reactions, occurring within hundredth or thousandth parts of a second. The alternating current oscillopolarographic method introduced by Heyrovsky in 1947 (cf. the monograph by Heyrovsky & Kalvoda, 1960) seems to be particularly suitable for studying the kinetics of reactions catalysed by caeruloplasmin.

The aim of the present work was to study the mechanism of caeruloplasmin action on serotonin, adrenaline, noradrenaline and ascorbic acid, and the effect of Fe^{2+} ion by the alternating current oscillopolarographic method.

MATERIAL AND METHODS

Caeruloplasmin was obtained from hog serum by the method of Holmberg & Laurell (1948). The final fraction was rendered free of traces of copper ion on Chelex-100 resin, dialysed for 12 hr. against 0.2 M-acetate buffer, pH 5.6, at 4°, and the solution concentrated on Sephadex G-25 to about 1.5% concentration. The obtained preparation was about 90% pure (as determined from the ratio of

extinctions at 610 and 280 mμ), the Cu/N ratio being 0.021. The amount of copper present in Cu^{2+} state was determined spectrophotometrically according to Peterson & Bollier (1955), and nitrogen by the colorimetric method of Burck (1960).

Oscillopolarographic procedure. To the polarographic cell, 5 ml. of 0.2 M-acetate buffer, pH 5.6, was introduced, then a minimum amount (0.1 - 10 μmoles) of the compound studied was added (control samples), quickly stirred, and 1 drop of about 1.5% aqueous solution of caeruloplasmin was added (proper samples). The temperature of the solutions was about 25°. The oscillograms were observed on the screen of the P-576 polaroscope (Křižik, Prague, Czechoslovakia). They were photographed from a distance of 40 cm. with an Exacta Varex camera.

Reagents. The 0.2 M-acetate buffer solution, pH 5.6, was prepared from acetic acid and sodium acetate, and freed of traces of heavy metal ions on a Chelex-100 column (Bio-Rad, Los Angeles, U.S.A.). Sephadex G-25 was from Pharmacia (Uppsala, Sweden). *p*-Phenylenediamine dihydrochloride (Fabryka Odczynników Chemicznych, Gliwice, Poland) was recrystallized twice from acetone and dried in dark vacuum desiccator over calcium chloride. The following reagents, A.R., were used without further purification: acetic acid, sodium acetate, $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$ and EDTA (Fabryka Odczynników Chemicznych, Gliwice); serotonin creatinine sulphate complex, and crystalline L-adrenaline (Merck, Darmstadt, Germany); L-noradrenaline (K. & K. Laboratories, Jamaica, N.Y., U.S.A.); L-ascorbic acid (Roche Products, Welwyn Garden, England).

RESULTS

The oscillograms shown in the photographs reflect the electrode processes of the substances studied, on the dropping mercury electrode polarized by alternating current. For qualitative characterization of the depolarizer, i.e. the substance lowering within a certain range of potentials the ability of the dropping electrode to become polarized, Q values were calculated from the photographs. Q corresponds to the ratio of the distance of indentation from the left marginal point to the whole distance between both marginal points (Heyrovsky & Kalvoda, 1960).

The characteristic oscillogram of native caeruloplasmin (Fig. 1) had two symmetrical indentations, one in the cathodic (upper) part and the second in the anodic

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Plate I. Diagrams of oscillograms appearing on the screen of the P-576 Polaroscope (Křižik, Prague) in the system of co-ordinates $\frac{dE}{dt} - f(E)$; the photographs were taken immediately or within a few seconds after addition of the respective components to the polarographic cell which contained 5 ml. of 0.2 M-acetate buffer, pH 5.6; temperature 25°. Fig. 1. Caeruloplasmin (0.2 m-mole Cu). Fig. 2. EDTA (10 μmoles). Fig. 3. Ascorbic acid (50 μmoles) and Fe^{2+} (0.4 μmole). Fig. 4. Caeruloplasmin and sulphuric acid (20%, a few drops). Fig. 5. Caeruloplasmin and EDTA.

Plate II. Diagrams of oscillograms appearing on the screen of the P-576 Polaroscope (Křižik, Prague). Conditions as in Plate I. Concentration of the components: caeruloplasmin, 0.2 m-mole Cu; Fe^{2+}, 0.4 μmole; Fe^{3+}, 0.4 μmole; phenylenediamine, 10 μmoles; serotonin, 1 μmole; adrenaline, 1 μmole; noradrenaline, 1 μmole; ascorbic acid, 50 μmoles.

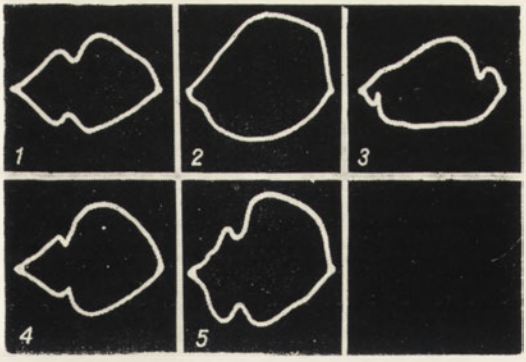
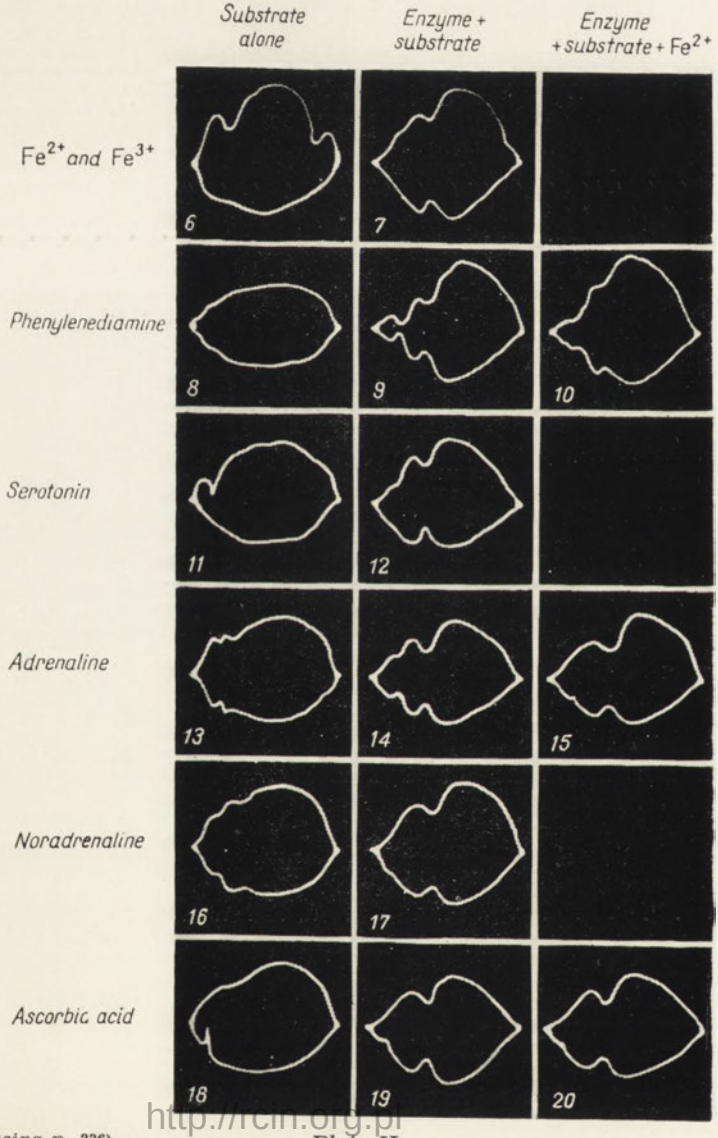


Plate I



Z. Prasal (*facing p. 236*).

Plate II

(lower) part, the Q value being 0.4. These well-defined indentations appeared immediately after addition of caeruloplasmin to the buffer solution. In connection with the controversial views on the binding of caeruloplasmin copper by EDTA (Humoller, Mockler, Holthaus & Mahler, 1960; Blumberg, Eisinger, Aisen, Morell & Scheinberg, 1963) it seems of interest that the oscillogram of caeruloplasmin in the presence of EDTA exhibited the same well preserved indentations (Fig. 5), suggesting that under the conditions used there was practically no chelation by EDTA.

Caeruloplasmin denatured by addition of a few drops of 20% sulphuric acid gave an oscillogram (Fig. 4) with more sharply defined indentations and a less streamlined outline in comparison with the oscillogram of the native enzyme. However, the position and depth of indentations remained almost unaltered, which seems to indicate that the indentations derive from copper ions.

Figure 9 illustrates the reaction of caeruloplasmin with *p*-phenylenediamine, a substrate commonly used for colorimetric estimation of caeruloplasmin. Symmetrical indentations appeared on the oscillographic curve and became better defined with time; they corresponded to an oxidation product with a Q value lower than that of caeruloplasmin.

On the oscillogram of the mixture of ferrous sulphate and ferric chloride in buffer solution (Fig. 6) the indentations produced by Fe^{2+} ion are visible in the right section of the cathodic part of the curve, and those produced by Fe^{3+} in the left section. On addition of caeruloplasmin these indentations disappeared (Fig. 7). In the system containing Fe^{2+} ion, *p*-phenylenediamine and caeruloplasmin, the indentations were less sharply defined and their distance from potential axis was greater (Fig. 10).

The effect of Fe^{2+} ion on oxidation by caeruloplasmin of some compounds naturally occurring in blood, was then studied. Serotonin alone gave a single sharp indentation with a Q value of 0.13 (Fig. 11); on addition of the enzyme the depth of this indentation decreased markedly (Fig. 12). Adrenaline was characterized by two double indentations (Fig. 13), of which that in the anodic part was better defined. In the presence of caeruloplasmin the depth of the anodic indentation increased and the distance of either indentation from potential axis was reduced by a half (Fig. 14). Addition of Fe^{2+} ion (0.4 μmole) brought the shape of the curve (Fig. 15) almost to that for the enzyme alone (cf. Fig. 1). The oscillogram of noradrenaline (Fig. 16) had a double weakly defined indentation in either part of the curve. The anodic indentation nearer to the left marginal point was better defined. On addition of caeruloplasmin the indentation disappeared and the shape of the curve (Fig. 17) corresponded to the oscillogram of the enzyme alone better than it was the case with adrenaline. The indentation corresponding to ascorbic acid with a Q value of 0.15 (Fig. 18) became much weaker when caeruloplasmin was added (Fig. 19). On addition of Fe^{2+} to ascorbic acid, the indentation remained unaltered (Fig. 3). When, however, caeruloplasmin was added to the solution containing both ascorbic acid and Fe^{2+}, the indentations of ascorbic acid and Fe^{2+} disappeared (Fig. 20) and the oscillogram became almost identical with that for the enzyme alone (cf. Fig. 1).

DISCUSSION

Studies on reversible decolorization of caeruloplasmin (Curzon, 1963, 1965), studies on the number and state of copper atoms in the enzyme by the electron spin resonance technique (Blumberg *et al.*, 1963; Malmström & Neilands, 1964) and on enzymic oxidation of Fe^{2+} ion (Osaki & Walaas, 1967) suggested the reversibility of the oxido-reductive properties of caeruloplasmin.

The observations described in the present communication, concerning the behaviour of caeruloplasmin alone and in the presence of substrates, carried out by the oscillopolarographic technique, have given the first direct electrochemical evidence that caeruloplasmin forms a strictly reversible oxidation-reduction system, with a Q value of 0.4 under the conditions used. This is evidenced by the fact that the anodic and cathodic indentations are symmetrical, have the same potential (Figs. 1, 20) and appear instantaneously.

One molecule of caeruloplasmin contains eight atoms of copper: four Cu^{2+} and four Cu^{1+} (Scheinberg & Morell, 1957; Blumberg *et al.*, 1963). The enzymic activity and blue colour are dependent on the specifically bound Cu^{2+} atoms, as indicated by the hyperfine electron spin resonance spectra (Broman, Malmström, Aasa & Vänngård, 1962). During the action of the enzyme, the Cu^{2+} ions are reduced to Cu^{1+} which are re-oxidized by O_2 (Broman, Malmström, Aasa & Vänngård, 1963). The function of the remaining four Cu^{1+} is not fully elucidated. They may participate in binding of substrate (Broman *et al.*, 1963) and remain in interaction with Cu^{2+} (Marriott & Perkins, 1966). It seems that a grouping of four Cu^{2+} and four Cu^{1+} atoms forms the basis of the reversible oxidation-reduction system of caeruloplasmin. Thiol groups are probably not involved, as one molecule of caeruloplasmin contains only one thiol group (Kasper & Deutsch, 1963), and the oscillogram of caeruloplasmin does not exhibit any increase in the number of indentations after denaturation with sulphuric acid (Fig. 4).

As the mechanism of the electrode processes occurring in the systems of caeruloplasmin with Fe^{2+} ion, *p*-phenylenediamine, ascorbic acid, serotonin, adrenaline and noradrenaline is not simple, and the oxido-reductive reactions may be complicated by adsorption of the glycoprotein enzyme, the results should be interpreted with circumspection. In the oscillograms, the outline characteristic of caeruloplasmin is maintained after the addition of substrate (Figs. 7, 9, 12, 15, 17 and 20); the deformations may be different, being the greatest with *p*-phenylenediamine (Fig. 9) and the smallest with noradrenaline (Fig. 17). It is difficult to say whether these deformations correspond to enzyme-substrate complexes or are due to possible adsorption of caeruloplasmin on the dropping electrode, causing changes in its capacity. It should be noted, however, that on addition of Fe^{2+} ion the deformations disappear (Figs. 15, 20) and the oscillogram becomes almost identical with that for the enzyme alone (Fig. 1). It seems that this phenomenon may be explained by the accelerating effect of Fe^{2+} on the oxido-reductive reactions of caeruloplasmin. The formed reaction products, which are polarographically inactive (eg. Fe^{3+}, dehydroascorbic acid) dissociate from the complex liberating the enzyme. The

kinetic studies of Osaki (1966) and Osaki & Walaas (1967) have demonstrated that Fe^{2+} ion not only stimulates the activity of caeruloplasmin but is also its most effective substrate. The results of our study are in agreement with this conception. The reversible oxidation-reduction system of caeruloplasmin through mediation of Fe^{2+} ion may oxidize the substances of positive or negative electrode character present in the blood. For a more detailed evaluation of the observed phenomena further experiments are required.

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OSCYLOPOLAROGRAFICZNE BADANIE WŁASNOŚCI OKSYDO-REDUKCYJNYCH CERULOPLAZMINY

Streszczenie

1. W oparciu o oscylopolarografię zmiennoprądową (A.C. Oscillopolarographic Method) i przy zastosowaniu polaroskopu Křižik P-576 z kroplową elektrodą rtęciową dokonano obserwacji procesów elektrodowych ceruloplazminy.

2. W 0,2 M buforze octanowym o pH 5,6 i temp. 25° wykryto w strukturze ceruloplazminy istnienie odwracalnego układu oksydo-redukcyjnego o wartości $Q = 0{,}4$.

3. Potwierdzono charakter aktywujący jonów Fe^{2+} w procesach oksydo-redukcyjnych ceruloplazminy z substancjami biologicznie czynnymi.

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RIBOFLAVIN FLAVOPROTEIN FROM EGG YOLK

ANALYTICAL AND BIOPHYSICAL DATA

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1. The riboflavin flavoprotein preparation isolated from egg yolk was homogeneous on column chromatography, free-boundary electrophoresis, disk electrophoresis, immunoelectrophoresis, and on sedimentation in ultracentrifuge. Its isoelectric point is at pH 4.16 and electrophoretic mobility at pH 7.2 is 15.76×10^{-5} cm.^{2}v^{-1}sec.$^{-1}$. 2. The molecular weight of the flavoprotein, determined from sedimentation data, diffusion coefficient and partial specific volume, is 36 000. 3. The flavoprotein is a glycoprotein possessing per one molecule about 297 amino acid residues, 21 sugar residues and 1 riboflavin residue. It is probably a dimer composed of two identical polypeptide chains, with histidine as the *N*-terminal amino acid. 4. On the basis of the observed differences between flavoprotein and apoprotein in the number of potentiometrically titratable groups, the binding of protein to flavin has been discussed.

In the course of our studies on the naturally occurring complexes of vitamins with proteins, a riboflavin flavoprotein has been isolated from hen's egg yolk, and some of its properties reported (Ostrowski, Skarżyński & Żak, 1962; Ostrowski & Krawczyk, 1963; Żak & Ostrowski, 1963; Weber, Żak & Ostrowski, 1966). A flavoprotein possessing similar properties has been isolated from egg white by Rhodes, Bennett & Feeney (1959). The egg-yolk flavoprotein is a glycoprotein of mol. wt. 36 000, containing one molecule of riboflavin per one molecule of protein (Ostrowski & Krawczyk, 1963). The flavoprotein does not undergo reduction on the dropping mercury electrode (Żak & Ostrowski, 1963) but is reduced by hydrosulphite or dithionite (Żak & Steczko, in preparation). The reduced form is reoxidized in the presence of molecular oxygen or ferricytochrome *c*. Riboflavin can be released from the flavoprotein by dextran-gel filtration at pH 3.0. The obtained apoprotein retains the ability to bind flavin, giving then the same absorption spectrum as the native flavoprotein (Żak & Ostrowski, 1963).

In the present paper, criteria of homogeneity of the flavoprotein are discussed, and its amino acid and sugar composition and other results pertaining to the primary structure of the protein are reported.

MATERIALS AND METHODS

The flavoprotein was obtained from fresh hen's egg yolk as described previously (Żak & Ostrowski, 1963). After final purification of the protein on DEAE-cellulose column, the bright yellow solution was dialysed against water and concentrated on a small DEAE-cellulose column. The obtained preparation was stored at -20° and protected from light.

To obtain apoprotein, 10 - 20 mg. of the flavoprotein in 1 ml. of 0.1 M-citrate buffer, pH 3.0, was applied to a Sephadex G-25 column (1.5×30 cm.) and eluted with the same buffer at 3° (Żak & Ostrowski, 1963). The apoprotein was then dialysed against water and freeze-dried.

Monovalent and polyvalent antisera were obtained in rabbits as described by Weber *et al.* (1966) by injection of either a pure or partially purified flavoprotein preparation.

Special reagents: DEAE-cellulose (Serva, Heidelberg, Germany); DEAE-Sephadex A-25 (medium), Sephadex G-25 and G-100 (Pharmacia, Uppsala, Sweden); Dowex 2X8 (Fluka A. G., Buchs S. G., Switzerland); trypsin, three times crystallized (Sigma, Biochem. Corp., St. Louis, Mo., U.S.A.); neuraminidase from *Vibrio cholerae* (activity 500 units/ml.), sodium dodecyl sulphate (SDS) and fluorodinitrobenzene (Light & Koch, Colnbrook, England). Acid phosphomonoesterase was isolated from human prostate as described by Ostrowski & Tsugita (1961). Sugars used as standards were products of: fucose and glucosamine, L. Light & Koch; D-mannose and D-glucose, British Drug Houses (London, England); D-galactose, Pfanstiehl Chem. Co. (Wankegan, Ill., U.S.A.). Agar gel (Bacto-Agar, Difco, Detroit, U.S.A.) was purified according to Grabar & Burtin (1960).

Moving-boundary electrophoresis. This was performed in an apparatus of C. Zeiss, model 35 (VEB, Jena, German Democratic Republic) at 150 v and 2.2 - 10.5 mA, at 4°. To determine the isoelectric point of the flavoprotein, acetate buffers of pH from 3.8 to 5.2, *I* 0.1, were used. Homogeneity of the preparations was checked at pH 8.6 in veronal buffer and at pH 7.2 in sodium phosphate buffer, both of *I* 0.1. In all experiments, the concentration of protein in the sample was 1%.

Electrophoresis on polyacrylamide gel. The separation was carried out by the method of Ornstein & Davis (1964) in a Canalco type apparatus (Canal Industrial Corp., Rockville, Ma., U.S.A.) on 7.5% polyacrylamide gel in tris-glycine buffer, pH 8.3, *I* 0.025, and a current of 3 mA per one gel column. Protein in the gel was stained with a 1% solution of amido black 10 B in 7% acetic acid, and the excess of the dye was removed by electrophoresis (Reisfeld, Lewis & Williams, 1962).

Immunoelectrophoresis. The method of Grabar & Williams (1955) in the modification of Scheidegger (1955) was used. Glass plates were covered with a thin layer of 1% agar gel in veronal buffer, pH 8.2, *I* 0.025. The separation was carried out at a potential gradient of 6 - 8 v/cm. for 30 - 60 min. After the precipitation arcs had formed, the plates were stained with amido black 10B by the technique described by Uriel (1960).

Ultracentrifugation. Analytical ultracentrifuge Spinco model E, provided with

standard schlieren optics was used. The sedimentation rate was determined at a protein concentration of 2 - 10 mg./ml., in potassium phosphate buffer, pH 7.0, *I* 0.1. The diffusion coefficient was determined in 0.1 M-sodium phosphate buffer, pH 7.0, according to Kahn & Polson (1947). The partial specific volume of flavoprotein was determined from the amino acid composition of the protein (Cohn & Edsall, 1943), taking into account the sugar component of the protein. From the obtained data, the molecular weight of flavoprotein was calculated.

Amino acid composition. The protein was dialysed against de-ionized water for about 70 hr., then the sample containing 5 mg. of protein was frozen at -70°, deaerated under vacuum and hydrolysed in a sealed glass tube with 6 N-HCl at 110° for 24, 44 and 70 hr. The hydrolysate was evaporated under vacuum in the presence of NaOH and P_2O_5, then dissolved in an appropriate volume of 0.2 M-citrate buffer, pH 2.2, and on 2-ml. sample of the obtained solution the analysis of the amino acid composition was made according to Spackman, Stein & Moore (1958) using the automatic Spinco model 120 B amino acid analyser.

For determination of cystine, 4.1 mg. of flavoprotein was oxidized with performic acid at -10° for 2.5 hr. according to Hirs (1956), then, after freeze-drying, the sample was hydrolysed as described above for 22 hr., and analysed in the automatic amino acid analyser. In the hydrolysate prepared in this way all cystine residues were oxidized to cysteic acid. Average results were calculated from two successive experiments.

To correct for degradation of some amino acids (serine, threonine) during hydrolysis, their content in the protein was calculated by extrapolation to zero-time hydrolysis. The concentration of most of the amino acids increased with time of hydrolysis and therefore the results obtained with the longest time of hydrolysis were taken for calculations.

Tryptophan was determined colorimetrically using *p*-dimethylaminobenzoic aldehyde according to the method of Spies & Chombers (1948) in the modification of Kupfer & Atkinson (1964).

Determination of N-*terminal amino acids.* The flavoprotein was treated with fluorodinitrobenzene (Fraenkel-Conrat, Harris & Levy, 1959) and DNP-amino acids were identified by paper chromatography in the following solvent systems: 1, *n*-butanol saturated with water; 2, *n*-butanol - butyl acetate - 1% NH_3 (1:2:3, by vol.); 3, *n*-butanol - acetic acid - water (4:1:5, by vol.) (Fraenkel-Conrat *et al.*, 1959). The course of the reaction of fluorodinitrobenzene with the protein was followed by measuring the utilization of NaOH using the Radiometer titrigraph (Copenhagen, Denmark).

Two-dimensional separation of peptides. Flavoprotein, 20 mg., was reduced with thioglycollate and carboxylated with iodoacetate according to Sela, White & Anfinsen (1959). The preparation was then diluted to 10 mg./ml. with 0.05 M-ammonium bicarbonate, pH 8, and 20 µl. of a trypsin solution (5 mg./ml. in 1 M-acetic acid) was added per 1 ml. of protein solution. After 16 min. at 37°, the reaction was stopped by adding 0.1 ml. of 1 M-acetic acid per 1 ml. The hydrolysate was dried in a vacuum desiccator over H_2SO_4 and NaOH, then dissolved in a small volume

of the buffer used for electrophoresis, and stored at -25°. For electrophoresis, 10 - 20 µl. of the solution containing about 2 mg. of the dry hydrolysate, was applied to Whatman no. 3 *MM* paper. The separation of peptides was carried out in the apparatus for high-voltage electrophoresis described by Ostrowski & Krawczyk (1966), in a buffer containing 15 ml. of acetic acid, 14 ml. of pyridine, and water up to 1000 ml., pH 4.7. After the electrophoretic run, the paper was air-dried and then the second separation was performed by descending chromatography perpendicular to that of the first run, using the system of *n*-butanol - acetic acid - water (4:1:5, by vol.). The electrochromatograms were stained by immersion into 0.5% ninhydrin solution in acetone, and then air-dried at room temperature until the colour appeared (about 10 hr.). Then the electrochromatograms were fixed with a 1% solution of cupric nitrate in acetone.

Protein determination. Protein was determined by measuring the extinction at 280 mµ in a Uvispec (Hilger & Watts, London, England) spectrophotometer, or by the method of Lowry, Rosebrough, Farr & Randall (1951). Nitrogen was determined by the Kjeldahl micromethod.

Analysis of the sugar composition. Preliminary estimations were carried out as follows: 50 mg. of flavoprotein was dissolved in 1 ml. of 0.5 N-HCl and hydrolysed in a sealed tube for 2 hr. at 110°. After cooling, the hydrolysate was twofold diluted with water and applied to a Sephadex G-25 column (85×0.9 cm.) equilibrated with 0.1 N-acetic acid. The elution was carried out with the same solution and 1-ml. fractions were collected at a rate of 10 ml. per hour. In each fraction, diluted with water to a volume of 2.5 ml., extinction was measured at 280 and 450 mµ, and the content of sugars was determined by the phenol method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) reading the extinction at 490 mµ. The sugar-containing fractions were pooled, concentrated under vacuum to a small volume and submitted to paper chromatography in the system of ethyl acetate - pyridine - water (10:4:3, by vol.) according to Jermyn & Isherwood (1949) and to paper electrophoresis in borate buffer, pH 8.6 (Galos & Ostrowski, 1954). The sugars were detected with the aniline-phthalate reagent according to Partridge (1949). Another sample of the flavoprotein was hydrolysed according to Caldwell & Pigman (1965) and the sugars were identified by paper chromatography with *n*-butanol - pyridine - water (5:3:2, by vol.) as solvent.

For quantitative analysis of sugars, the method of Walborg, Christensson & Gardell (1965) was used. The flavoprotein, 10 - 15 mg., was dissolved in 2 ml. of 1 N or 2 N-H_2SO_4, hydrolysed at 100° for 10 hr., neutralized using Dowex 2X8 (carbonate form), evaporated under vacuum and the dry residue dissolved in 1 ml. of 0.4 M-boric acid - 1 M-glycerol buffer, pH 6.8. One ml. of the hydrolysate was applied to a column (140×0.6 cm.) of Dowex 2X8 (200 - 400 mesh), equilibrated with the above buffer containing 0.05 M-NaCl, pH 6.8. The column was equipped with a water jacket to maintain the temperature at 50°. The elution was performed with the same buffer and fractions of 1 ml. were collected. The amount of sugar was determined with the aniline - acetate - phosphate reagent (Walborg & Christensson, 1965), measuring the extinction at 370 mµ. To identify the individual sugars

in the eluate, the column was calibrated under the same conditions with a standard mixture of sugars.

Glucosamine determination. About 2 mg. of the flavoprotein was heated at 110° with 20 ml. of water and 6 g. of Dowex 50 (H^+ form) (Munde & Verner, 1964). After 16 hr. the mixture was filtered and the resin washed several times with water, then treated 5 times with 2 N-HCl. The combined HCl solutions were evaporated under vacuum, the dry residue dissolved in a small volume of water and the amount of glucosamine determined using the Ehrlich reagent according to Rondle&Morgan (1955).

Sialic acid determination. Neuraminic acid was determined by the thiobarbituric method described by Warren (1959). The flavoprotein was hydrolysed with 5% trichloroacetic acid, 1 N-H_2SO_4, and by neuraminidase. A sample containing 2.5 - 5 mg. of protein was heated with 5% trichloroacetic acid or H_2SO_4 for 2 hr. at 60°, and after cooling sialic acid was estimated according to Warren. When neuraminidase was to be used for hydrolysis, 5 mg. of the flavoprotein was dissolved in 1 ml. of acetate buffer, pH 5.5 (0.05 M-sodium acetate - 0.1 M-NaCl - 0.01 M-$CaCl_2$, and HCl to pH 5.5), 25 units of neuraminidase was added, and incubation carried out at 5° for 10 days.

Determination of terminal groups of sugars. This was carried out by the periodate oxidation method of Fletcher, Marks, Marshall & Neuberger (1963). The flavoprotein, 2 μmoles, was dissolved in 10 ml. of water and an equal volume of 10 mM-$NaIO_4$ solution was added at 3 - 5°. The mixture was incubated at this temperature for several hours, samples being withdrawn at intervals to determine the utilization of periodate by iodometric titration with arsenite solution (Aminoff & Morgan, 1951).

Potentiometric titration of flavoprotein. This was performed in a pH-stat Radiometer (Copenhagen), model Titrator-Titrigraph $TTT_1/SBR_2/SBU_1$. Samples of about 10 mg. of protein were dissolved in 4 ml. of 0.15 M-KCl, brought to the isoelectric point (pH 4.16) and titrated with 0.2 N solutions of NaOH or HCl in a cell provided with a water-jacket, at 25°±0.05°. To denature the protein, it was dissolved in 0.15 M-KCl - 0.015 M-SDS solution, and after a few minutes the titration was carried out. Dephosphorylation of flavoprotein or apoprotein was carried out in the following way: to 19.2 mg. of flavo- or apoprotein dissolved in 2 ml. of 0.05 M-citrate buffer, pH 5.5, 20 μl. of acid phosphomonoesterase from human prostate was added (1 ml. of the enzyme solution split 1.158 m-moles of substrate during 1 min. at 25°; Ostrowski & Tsugita, 1961). The mixture was incubated for 2 hr. at 30°, then dialysed overnight against 0.15 M-KCl, diluted to the required volume with the same KCl solution, and submitted to titration.

RESULTS

Properties of flavoprotein

Criteria of chromatographic homogeneity of the flavoprotein. The preparation obtained on a preparative scale (from about 1000 egg yolks) according to the pro-

cedure described previously (Żak & Ostrowski, 1963) contained about 10% of chromatographically or electrophoretically detectable impurities. The ballast proteins were, however, easily separated by Sephadex G-100 gel filtration (Fig. 1). Yellow-coloured fractions emerging at 50 - 80 ml. of effluent which coincided with the second protein peak, were pooled and concentrated on a 2-ml. column of DEAE-cellulose equilibrated with 0.1 M-phosphate buffer, pH 5.8; the protein was eluted with 3 ml. of 0.5 M-NaCl - 0.1 M-phosphate buffer, pH 5.5. The concentrated solution contained several milligrams of flavoprotein in 1 ml. The flavoprotein was found to be homogeneous and free of detectable contaminations on DEAE-Sephadex A-25 chromatography and starch-block electrophoresis (Fig. 2). The spectrum of the purified preparation at pH 7.0 (Fig. 3) possesses three absorption maxima at 276, 375 and 458 mμ, the ratio of extinction at 276 and 458 mμ being 6.5. In the experiments described below, the flavoprotein preparation corresponding to this standard of purity was used.

Electrophoretic analyses. Electrophoresis on polyacrylamide gel at pH 8.3 (Fig. 4) demonstrated the homogeneity of the flavoprotein preparation, which appeared as a single sharp band migrating toward the anode. Even before staining the band was visible in the same place due to the yellow-coloured riboflavin.

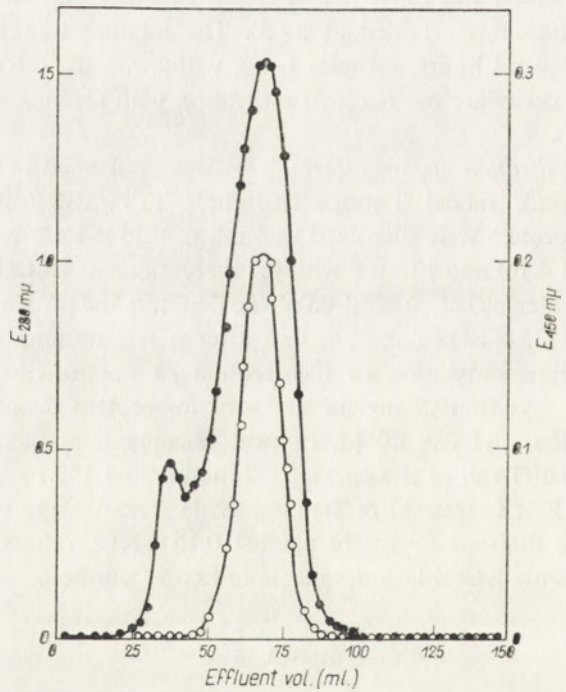


Fig. 1. Sephadex G-100 gel filtration of egg-yolk flavoprotein. A sample (30 mg.) of the protein after chromatography on DEAE-cellulose was dissolved in 1 ml. of 0.9% NaCl solution, applied to the column (125 × 1.4 cm.) and eluted with the same solution at a rate of 10 ml./hr. at 3°. Extinction was measured (●) at 280 mμ and (○) at 450 mμ.

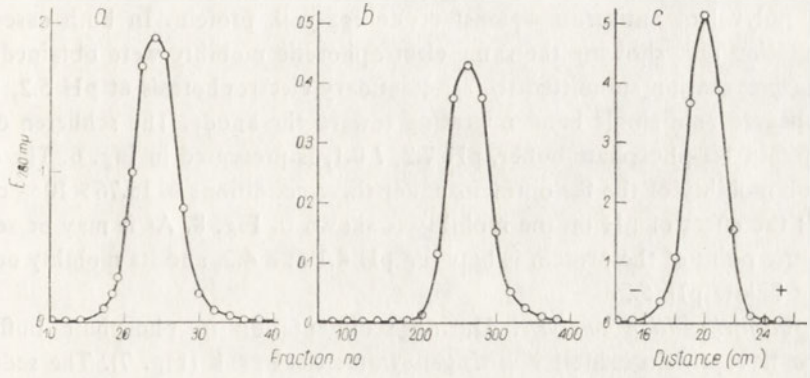


Fig. 2. Tests for homogeneity of the flavoprotein preparation. *a*, Chromatography on DEAE-cellulose column; conditions as described by Żak & Ostrowski (1963). *b*. Chromatography on DEAE-Sephadex A-25; 30 mg. of protein in 3 ml. of the starting buffer was applied to a column (30×4 cm.) which was prepared and then eluted as described by Żak & Ostrowski (1963). *c*, Electrophoresis on starch block (10×36 cm.) in 0.05 M-sodium phosphate buffer, pH 7.05. A sample containing 10 mg. of protein in 0.2 ml. of buffer was separated at 200 v, 35 mA for 6 hr. at 3°. Then the block was cut into 0.5 cm. segments, the protein eluted with water (3.5 ml.) and extinction measured at 280 mμ.

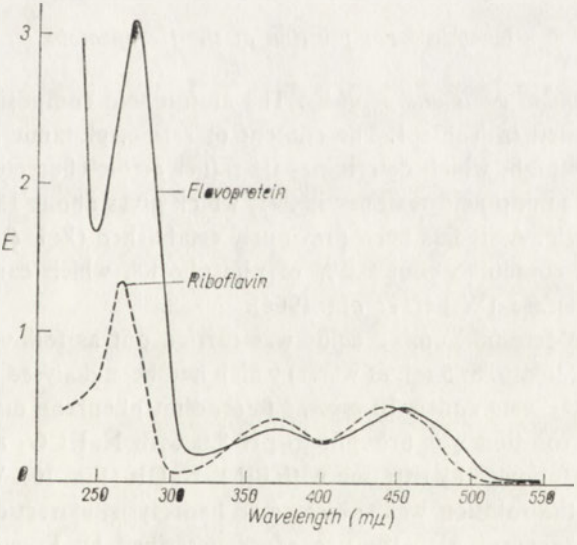


Fig. 3. Spectrum of (—) flavoprotein and (- - -) riboflavin dissolved in 0.05 M-sodium phosphate buffer, pH 7.0, obtained with the self-recording spectrophotometer SP-800 (Unicam, London, England). Concentration of protein 1.5 mg./ml., of riboflavin 0.0156 mg./ml.

Homogeneity of the preparation was confirmed also by immunoelectrophoresis carried out with a specific antiserum obtained against pure flavoprotein (Fig. 5) and a polyvalent antiserum against crude egg-yolk protein. In both cases single precipitation arcs showing the same electrophoretic mobility were obtained.

The preparation submitted to free-boundary electrophoresis at pH 5.2, 7.2 and 8.6 appeared as a single band migrating toward the anode. The schlieren diagram obtained in Na-phosphate buffer, pH 7.2, I 0.1, is presented in Fig. 6. The electrophoretic mobility of the flavoprotein under these conditions is 15.76×10^{-5} cm.^{2}v^{-1} sec.$^{-1}$; the effect of pH on the mobility is shown in Fig. 8. As it may be seen, the isoelectric point of the protein is between pH 4.1 and 4.2, and its mobility decreases rapidly below pH 5.2.

Ultracentrifugation analysis. During sedimentation in phosphate buffer, pH 7.0, the flavoprotein exhibited a single symmetrical peak (Fig. 7). The sedimentation coefficient decreased with the increase in protein concentration from 0.2 to 1%, giving no indication of any association-dissociation equilibrium (Fig. 9). The sedimentation coefficient, $S^0_{20,w}$, was found to be 3.16. The diffusion coefficient, D^0, determined for three concentrations of the flavoprotein (0.43, 1.13 and 1.5%) was 7.45×10^{-7} cm.^{2}sec.$^{-1}$. The partial specific volume of the flavoprotein, $\bar{V}$, is 0.699 ml./g. Hence the molecular weight of the flavoprotein was 36 000, calculated from the equation:

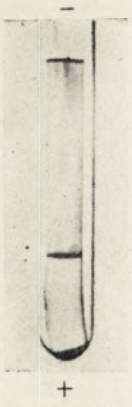
$$M = \frac{RT\, S^0_{20,w}}{D^0 (1 - \bar{V}\varrho)}$$

in which ϱ is the density of the solvent.

Chemical composition of the flavoprotein

Analysis of amino acids and peptides. The amino acid composition of the flavoprotein is presented in Table 1. The content of serine, glutamic acid and aspartic acid is especially high, which determines its rather acidic character (pI 4.16). The total number of amino acid residues is 297, which gives about 13% of nitrogen in a protein molecule. As it has been previously established (Żak & Ostrowski, 1963) the flavoprotein contains about 0.2% of phosphorus, which can be liberated by phosphomonoesterase (Weber *et al.*, 1966).

Analysis of *N*-terminal amino acids was carried out as follows: to 1 μmole of the flavoprotein (36 mg. in 5 ml. of water) which had been dialysed against bidistilled water free of CO_2, was added 10 mg. of fluorodinitrobenzene dissolved in 0.1 ml. of ethanol. The solution was brought to pH 8.0 with $NaHCO_3$ and the course of the reaction was followed by titration with 0.1 N-NaOH (Fig. 10). When the reaction was completed, the solution was subjected to hydrolysis, extraction and chromatographic analysis according to the procedure described by Fraenkel-Conrat *et al.* (1959). In all solvent systems applied, di-DNP-histidine was found to be the only DNP-amino acid present in the aqueous phase; its R_F values in the three successive solvents (see Methods) were 0.35, 0.50 and 0.57, respectively. In the ether phase



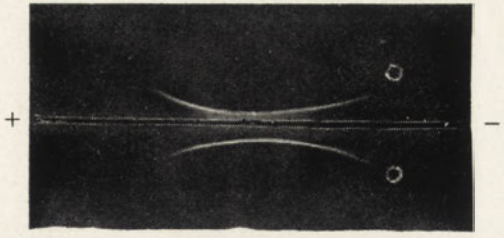


Fig. 4 Fig. 5

Fig. 4. Electrophoresis of flavoprotein on polyacrylamide gel. Conditions as described in Methods.

Fig. 5. Immunoelectrophoresis of flavoprotein using monovalent, undiluted rabbit antiserum. Conditions as described in Methods.

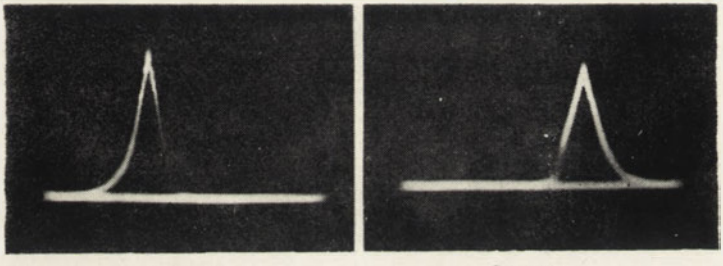


Fig. 6. Schlieren diagram of flavoprotein in 0.05 M-phosphate buffer, pH 7.2, 150 V, 8 mA, 27 min., temp. 4°.

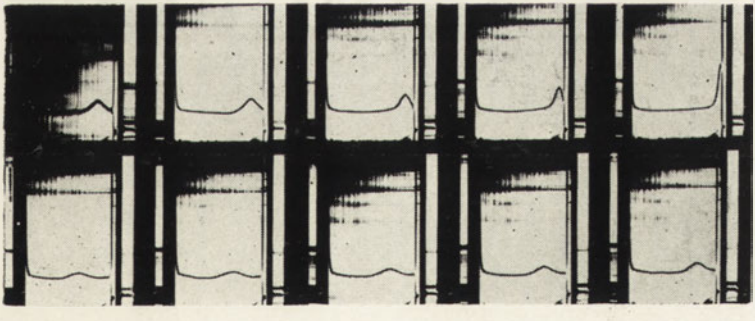


Fig. 7. Sedimentation (from right to left) of flavoprotein in the analytical ultracentrifuge. Concentration of protein 4 mg./ml., temp. 21.5°, bar angle 45°. The photographs were taken at 16 min. intervals, at 52 640 rev./min. Other conditions as described in Methods.

no DNP-amino acid has been detected. The presence of histidine as the *N*-terminal amino acid was confirmed by the phenylisothiocyanate method of Edman (1956).

To obtain further data on the primary structure of the flavoprotein, tryptic peptides (see Methods) were separated by high-voltage electrophoresis (Fig. 11). The repeated separations of peptides obtained at different enzyme to substrate ratios and varying time of hydrolysis, gave 15 - 17 well resolved ninhydrin-positive spots. Taking into account that each tryptic peptide should contain one lysine or arginine residue (Harris & Ingram, 1960), the number of the obtained peptides corresponds very closely to a half of the number anticipated from the amino acid analysis (22 lysine and 8 arginine, Table 1). This suggests that the flavoprotein may be a dimer composed of two identical subunits.

Analysis of sugars. The hydrolysate of flavoprotein obtained by heating the protein with 0.5 N-HCl in a sealed tube for 2 hr. at 110° and prepared as described in Methods, was separated on a Sephadex G-25 column (Fig. 12). Sugar-containing fractions (nos. 65 - 83) were pooled, concentrated, then hexoses and amino sugars

Table 1

Amino acid composition of flavoprotein from egg yolk

Amino acid	g./100 g. protein	g./36 000 g. protein	Number of residues
Lysine	7.67	3 143	21.5
Histidine	4.21	1 707	11.0
Arginine	3.76	1 499	8.6
Half cystine[a]	5.75	2 342	19.5
Aspartic acid	8.08	3 327	25.0
Threonine	3.05	1 309	11.0
Serine	11.51	5 118	48.7
Glutamic acid	12.34	5 046	34.3
Proline	3.21	1 347	11.7
Glycine	2.95	1 396	18.6
Alanine	3.33	1 479	16.6
Valine	2.86	1 218	10.4
Methionine	3.04	1 238	8.3
Isoleucine	2.95	1 233	9.4
Leucine	5.07	2 112	16.1
Tyrosine	4.86	1 975	10.9
Phenylalanine	3.35	1 321	8.0
Tryptophan[b]	3.99	1 572	7.7
Ammonia[c]	(3.26)	(1 173)	(69.0)
Total amino acids	92.08	32 954[d]	297.0

a Determined after oxidation of protein with performic acid.

b Determined colorimetrically.

c The content of amido groups is higher in relation to the number of dicarboxylic amino acids, probably due to the presence of carbohydrate chain in the protein (Marshall & Neuberger, 1960).

d Value obtained after subtracting $n - 1$ molecules of water and without taking into account the sugar component.

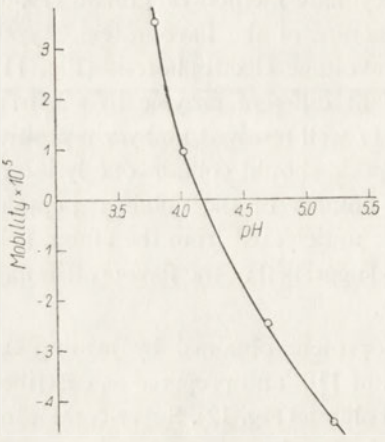


Fig. 8

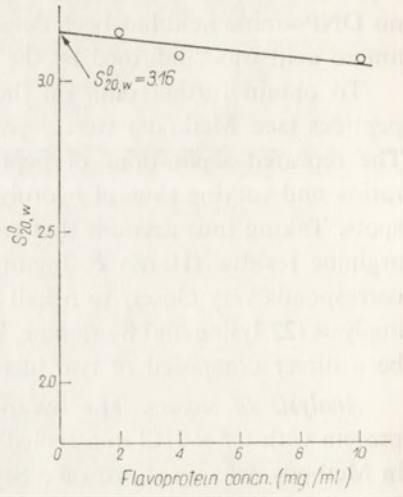


Fig. 9

Fig. 8. Effect of pH on the mobility of flavoprotein on free-boundary electrophoresis in acetate buffer, *I* 0.1. Conditions as described in Methods.

Fig. 9. Sedimentation coefficient of flavoprotein as a function of protein concentration. Conditions as for Fig. 7.

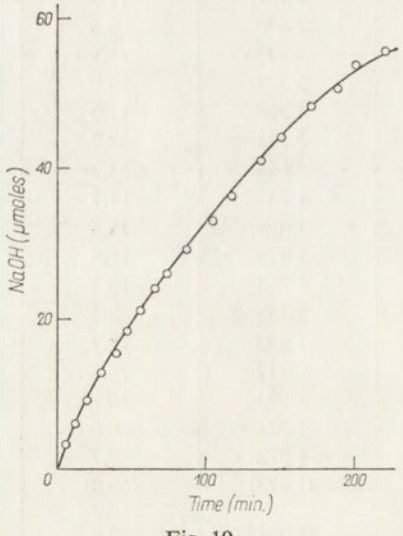


Fig. 10

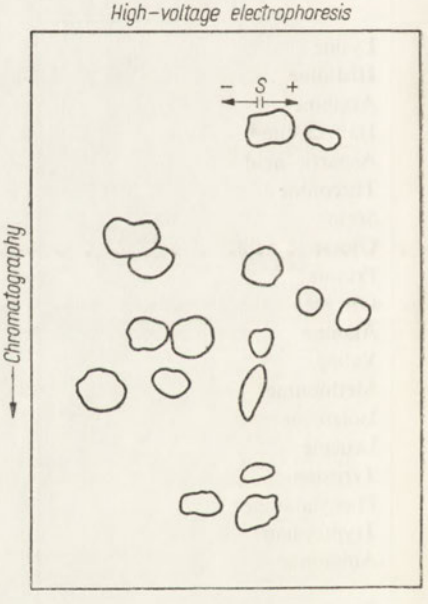


Fig. 11

Fig. 10. Course of alkali consumption (pH 8.0) during dinitrophenylation of flavoprotein at 40°.

Fig. 11. Two-dimensional separation of tryptic peptides. The flavoprotein was reduced with thioglycollate, carboxymethylated, and hydrolysed with trypsin. Separation in the first dimension: electrophoresis at 20 v/cm., 60 mA, 2.5 hr.; in the second dimension: descending chromatography in *n*-butanol - acetic acid - water (4:1:5, by vol.).

were identified by paper chromatography and electrophoresis. The latter component was also found on analysis of the amino acid composition. Since the conditions under which the above hydrolysis of the flavoprotein was carried out did not lead to complete separation of sugars from protein, in further experiments the hydrolysis was carried out in 1 N or 2 N-H_2SO_4 at 100° for 10 hr. (Walborg *et al.*, 1965). The

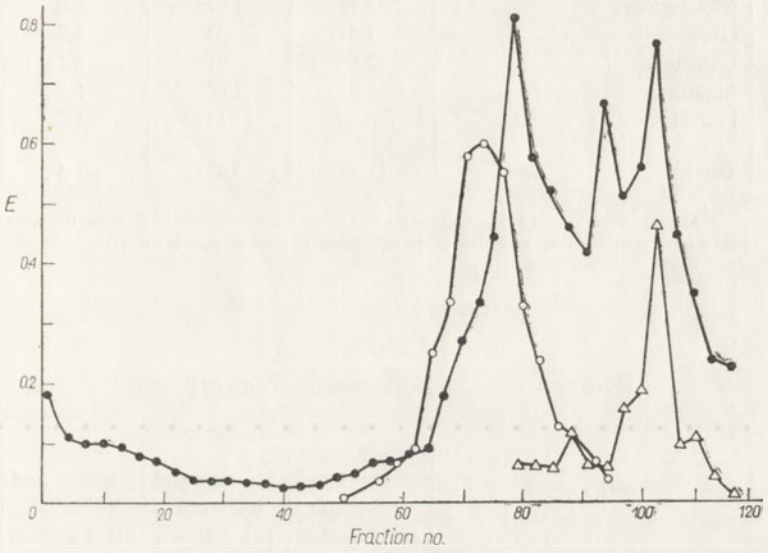


Fig. 12. Separation of sugars by Sephadex G-25 gel filtration of flavoprotein acid hydrolysate. Conditions as described in Methods. (○), Extinction at 490 mμ (determination of sugars with phenol reagent); (●), extinction at 280 mμ; (△), extinction at 450 mμ (flavin derivatives).

hydrolysate was neutralized with $Ba(OH)_2$ and the excess of barium ion was removed with gaseous CO_2. The neutral clear solution was dried under vacuum, the residue dissolved in warm ethanol and evaporated again. The sediment was dissolved in a small volume of water and submitted to paper chromatography (Fig. 13). Five sugars were identified: mannose, galactose, glucose, fucose and glucosamine.

An example of quantitative separation of neutral sugars of the flavoprotein on analytical Dowex 2X8 column by the method of Walborg *et al.* (1965) is shown in Fig. 14. The flavoprotein was found to contain a rather large amount of mannose and galactose. The total number of sugar residues in the flavoprotein amounts to about 21 per 1 mole of protein (Table 2).

Analysis of the flavoprotein for the presence of sialic acid demonstrated that, irrespective whether hydrolysis was carried out in acidic medium or using neuraminidase, the values from 4 determinations amounted to 0.32 - 0.50 mole of sialic acid per 1 mole of protein. Hence it appears that sialic acid is not an integral component of flavoprotein but it is found only as a result of minute contamination by other egg proteins, in which sialic acid is in high concentration.

Table 2

Carbohydrate composition of flavoprotein from egg yolk

The carbohydrates were separated on Dowex 2X8 (Fig. 14).

Sugar	g./100 g. protein	g./36 000 g. protein	Number of residues
Glucosamine[a]	3.99	1 075	6.0
Glucose	1.61	576	3.2
Galactose	2.65	919	5.1
Mannose	2.95	1 063	5.9
Fucose	0.58	197	1.2
Total	11.76	3 445	21.4

[a] Average values from 4 determinations, two of which were carried out using the automatic amino acid analyser, and two by the colorimetric method (see Methods).

Table 3

Titration data of flavoprotein from egg yolk

Protein	Ionizable groups		
	Carboxyl and phosphate pH 2.0 - 5.5	α-amino and imidazole pH 5.5 - 8.0	ε-amino and phenolic pH 8.0 - 11.0
Flavoprotein	68	14	5
Apoprotein	82	17	29
Dephosphorylated flavoprotein	21	23	12
Dephosphorylated flavoprotein in the presence of SDS	34	36	2

Table 4

Total composition of amino acids, carbohydrates and flavin of flavoprotein from egg yolk

Compounds	g./100 g. protein	g./36 000 g. protein	Number of residues
Amino acids	92.08	32 954	297.0
Carbohydrate	11.78	3 445	21.4
Riboflavin[a]	1.04	376	1.0
Total	104.90	36 775	319.4

[a] Riboflavin was determined as described by Żak & Ostrowski (1963).

The course of periodate oxidation of terminal groups of flavoprotein sugars is shown in Fig. 15. The reaction appears to be biphasic, the first step being rapid and the second much slower. After 22 hr. of incubation with periodate at about 4°, over 17 μmoles of periodate was reduced; on extrapolation to zero time this value gives 12 molecules of $NaIO_4$ per one molecule of the flavoprotein. The amount

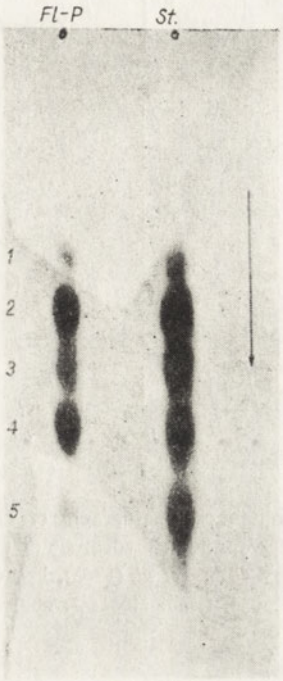


Fig. 13. Identification of sugars of the flavoprotein by chromatography on Whatman no. 1 paper with *n*-butanol - pyridine - water (5:3:2, by vol.). The spots were located with the aniline-phthalate reagent. *Fl-P*, flavoprotein hydrolysate; *St*, standards: *1*, glucosamine; *2*, galactose; *3*, glucose; *4*, mannose; *5*, fucose.

of periodate may indicate that the carbohydrate moiety in the flavoprotein studied is linear or has only one branch point (Hough & Taha, 1956).

Potentiometric titration. Titration curves for the native flavoprotein, for apoprotein obtained by splitting of riboflavin at pH 3.0, and after dephosphorylation and denaturation with SDS, are shown in Figs. 16 and 17. The titration data were calculated and expressed graphically according to Kenchington (1960) and Breslow & Gurd (1963). The pH ranges at which the number of ionizable groups were calculated, were determined on the basis of intrinsic dissociation constants obtained for different proteins and summarized by Steinhardt & Zaiser (1955). From Fig. 16 it may be seen that splitting of riboflavin caused significant changes in the shape of the curve of potentiometric titration of the flavoprotein. Quantitative data are presented in Table 3 and it is apparent that throughout the range of the pH values tested, a greater number of groups was accessible in the apoprotein than in the flavoprotein; at acidic pH values this difference amounted to 14 groups (α, β, γ-carboxylic groups and phosphate groups), at pH 5.5 - 8.0 three masked imidazole groups (or terminal α-amino groups) were liberated and at alkaline pH values an

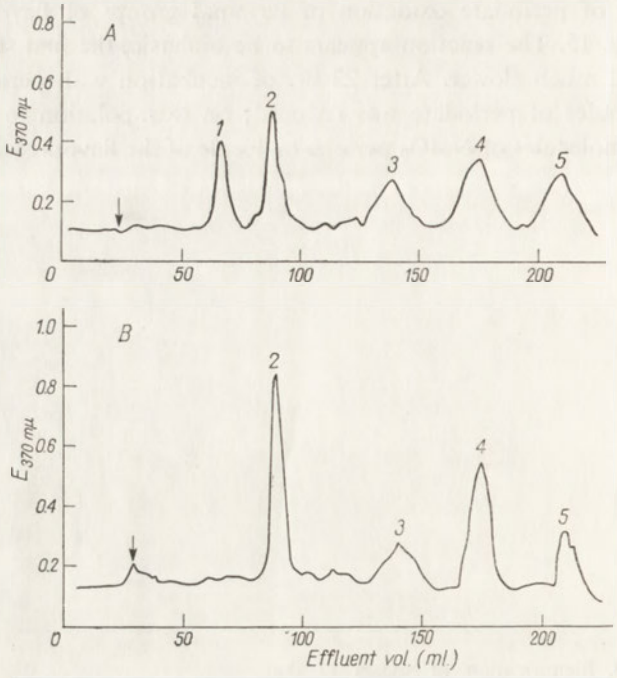


Fig. 14. Column chromatography on Dowex 2X8 of (*A*), standard sugars, and (*B*), sugars released from the flavoprotein. Hydrolysis of the flavoprotein and separation were carried out at 50° according to Walborg *et al.* (1965). For details see Methods. *1*, Rhamnose; *2*, mannose; *3*, fucose; *4*, galactose; *5*, glucose. The arrow indicates the void volume.

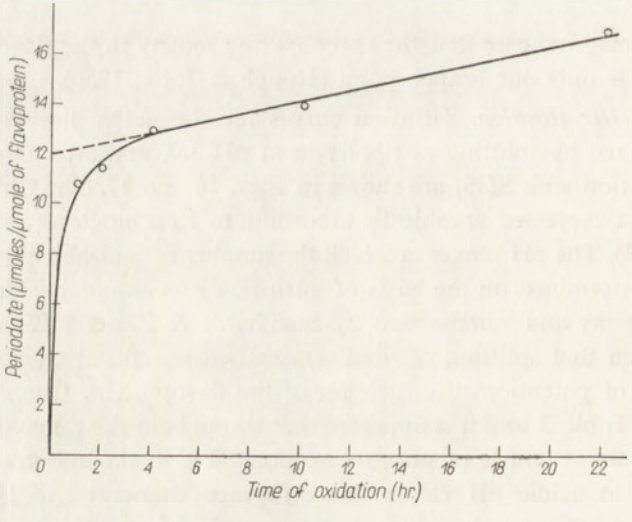


Fig. 15. The course of periodate oxidation of sugar terminal groups of flavoprotein.

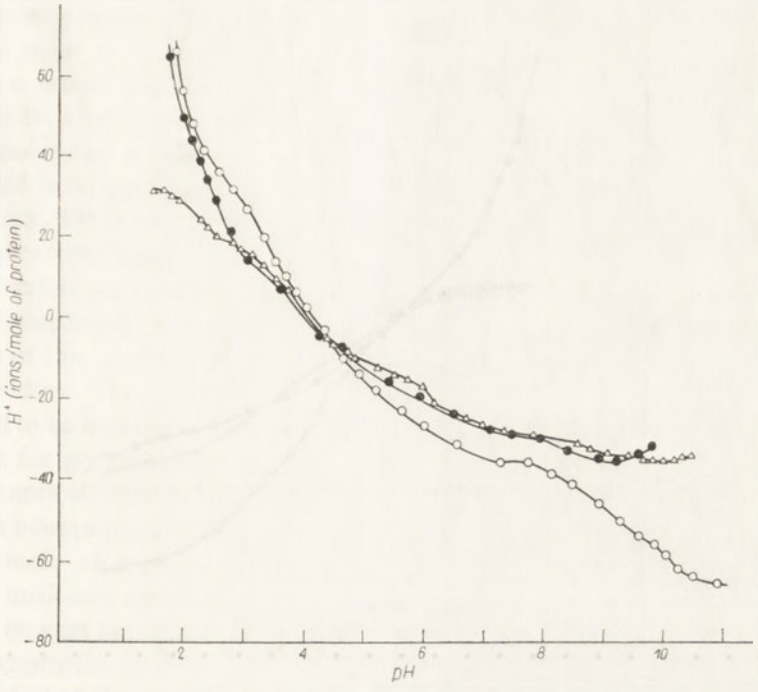


Fig. 16. Potentiometric titration. The protein, 9.6 mg. in 4 ml. of 0.15 M-KCl, was brought to the isoelectric point and titrated in two directions with NaOH and HCl at 25°. (●), Flavoprotein; (○), apoprotein; (△), dephosphorylated apoprotein.

increase of 24 groups (ε-amino and phenolic groups) was observed. For native flavoprotein the inflection points appeared at pH 3 and 8, whereas for apoprotein at pH 3.2 and 7.8. Dephosphorylation by phosphomonoesterase caused, both in flavoprotein and apoprotein, a decrease in the number of ionizable groups at pH 2 - 5.5, and an increase in imidazole groups and groups titratable at pH 8 - 11. Dephosphorylation of flavoprotein and denaturation with SDS caused a small decrease in acidic and a large increase in imidazole groups (Fig. 17) in comparison with the native protein. The presence of SDS caused an increase in acidic groups in relation to the dephosphorylated flavoprotein, which is in agreement with observations on titration of other proteins (Sparks, Da Costa & Friedberg, 1961). SDS caused also an increase in the number of groups titratable at pH 5.5 - 8 in comparison with the protein which had been dephosphorylated but not denatured, which simultaneously leads to masking of ε-amino and phenolic groups. To interpret the behaviour of the flavoprotein during titration in the presence of SDS a more complete analysis is necessary.

The data obtained by titration are not in full agreement with the results of the analysis of amino acid composition (cf. Table 1), especially as regards the groups titratable at pH 5.5 - 8.0. This discrepancy is probably due to the fact that the flavoprotein is a glycoprotein possessing a carbohydrate moiety which undoubtedly

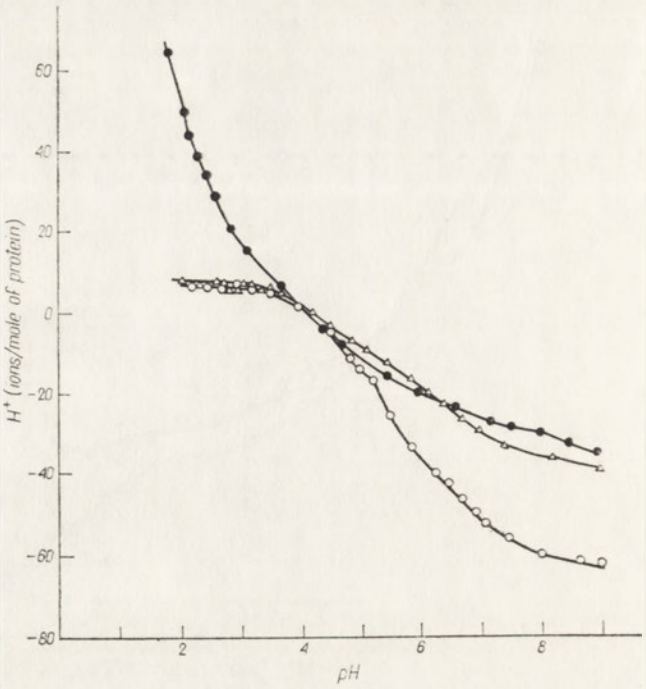


Fig. 17. Potentiometric titration of (●), flavoprotein, (△), dephosphorylated flavoprotein and (○), flavoprotein dephosphorylated in the presence of SDS. Conditions as for Fig. 16.

has a masking effect on the ionizing groups of the protein. Moreover, the presence of strongly acidic phosphate groups affects the number of titrated carboxyl as well as α-amino and imidazole groups (Fölsch & Österberg, 1959).

DISCUSSION

The flavoprotein obtained from egg yolk is a specific conjugated protein containing riboflavin as a prosthetic group. The dissociation coefficient at pH 7.0 determined on the basis of spectrofluorometric estimations, is 2.65×10^{-9} M (Ostrowski & Krawczyk, 1963) and indicates a rather strong binding between protein and flavin. The ratio of flavin to protein, calculated from spectrophotometric, spectrofluorometric and amperometric titration data and from microbiological analysis, is 1:1 (Żak & Ostrowski, 1963).

The flavoprotein preparation obtained according to the previously described method (Żak & Ostrowski, 1963) contained 10% of contaminations. Freeze-drying in this procedure is omitted, as it probably leads to aggregation of flavoprotein molecules. In the present work, using filtration on dextran gel a homogeneous preparation was obtained which corresponded to the standard of purity accepted in this work.

The homogeneous flavoprotein is stable over the pH range 3.5 - 8.5 and its isoelectric point is between pH 4.1 and 4.2. The acidic character of the protein is due to a rather large content of dicarboxylic amino acids (Table 1). At pH 3.0 flavin can be separated from the protein by dialysis or filtration on dextran gel.

The molecular weight of the flavoprotein determined in the present work is 36 000, and is in good agreement with the value determined previously (Ostrowski & Krawczyk, 1963) by the sedimentation equilibrium method of Ehrenberg (1957), and by ultracentrifugation in a sucrose gradient with horse oxyhaemoglobin and ox-liver catalase as standard proteins (Żak & Ostrowski, 1963).

The flavoprotein is a glycoprotein containing about 11% of carbohydrates; one mole of this protein contains about 14 hexose, 1 methylpentose and 6 aminohexose residues. The calculated value of partial specific volume of the flavoprotein was found to be between the corresponding values for the majority of simple proteins and those for glycoproteins. In the carbohydrate moiety of the flavoprotein, only trace amounts of sialic acid were found, both after acid hydrolysis and after treatment with neuraminidase. The analysis gave a value of less than half a mole of sialic acid per mole of protein. After treatment with neuraminidase, the flavoprotein exhibited unaltered mobility on paper electrophoresis and unaltered immunological reactivity on agar gel (Weber *et al.*, 1966). Preliminary results on periodate oxidation of the flavoprotein seem to indicate, if the interpretation of Hough & Taha (1956) is accepted, that the carbohydrate moiety is linear or possesses not more than one branch point. However, this conclusion is only tentative because the course of oxidation was determined only on the basis of periodate utilization, whereas no quantitative estimations were made of formic acid and oxidized carbohydrate derivatives arising as reaction products.

The amino acid composition indicates that the polypeptide of the flavoprotein is composed of 41 basic amino acid, about 60 acidic, 60 aliphatic hydroxylamino acid, about 90 neutral aliphatic amino acid, 22 aromatic amino acid, 12 proline and 19 cystine residues. Thus the protein studied is composed approximately of 297 amino acids and 21 monosaccharides (cf. Table 4), the content of serine and glutamic acid being especially high.

The analysis of terminal amino acids was only qualitative and incomplete. Nevertheless it seems that histidine is the *N*-terminal amino acid.

Results of the analysis of peptides of the flavoprotein by the fingerprint method after reduction of disulphide bonds, suggest that under these conditions the protein dissociates into two identical subunits. The number of tryptic peptides which were found on the electrochromatogram and which should contain either an arginine or a lysine residue, was approximately equal to a half of the number expected on the basis of the amino acid composition and molecular weight. There is a possibility that the method applied in the present work gave somewhat inaccurate results; first, the peptides might have been incompletely separated and some spots could contain more than one peptide; secondly, it could happen that not all protein was digested by trypsin and that some peptides could remain in the limit polypeptide. However, after hydrolysis by trypsin of the reduced protein no insoluble material

was detected. Inadequate separation of peptides also seems to be excluded because the low molecular weight of the flavoprotein points to the presence of about 30 peptides, and repeated electrophoretic separations of a mixture of peptides at different pH values and chromatography for different time intervals did not result in any increase in the number of ninhydrin-positive spots. Therefore it seems possible to conclude that the flavoprotein is composed of two subunits.

Titration of the flavoprotein not only permits to gain better knowledge of the structure of the protein itself but moreover it may give indications as to the mechanism of binding of riboflavin. Titration at pH values below 7.0 may show whether the presence of riboflavin influences the course of proton retention during titration of the protein in the absence of flavin which, as it has been demonstrated, is involved in liberation of the masked ionizable groups.

From the titration data it appears that the native flavoprotein containing 68 acidic and 19 basic ionizable groups behaves like a polyelectrolyte of a globular structure, possessing at zero charge a tight random coil configuration (Tanford, 1958). Splitting of riboflavin liberates the acidic, imidazole, ε-amino and phenolic groups, and the apoprotein has a negative charge over almost the whole range of titration (Fig. 16). These data indicate that at least a part of the groups which are unmasked after splitting of riboflavin participate in its binding with the protein. Since splitting of riboflavin occurs already at pH 3.0, it is probable that labile covalent or electrostatic bonds are involved, which at this pH value undergo dissociation making numerous groups accessible to titration. This would confirm the view that flavin in the flavoprotein is bound by a number of protein groups and the isoalloxazine ring (Theorell & Nygaard, 1954; Rhodes *et al.*, 1959; Ehrenberg, 1962; McCormick & Butler, 1962), influencing to a marked degree the spatial configuration of the apoprotein (Kotaki, Naoi & Yagi, 1966; Yagi, Naoi & Kotaki, 1966).

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RYBOFLAWINOWY FLAWOPROTEID Z ŻÓŁTKA JAJA

DANE ANALITYCZNE I BIOFIZYCZNE

Streszczenie

1. Otrzymany ryboflawinowy flawoproteid z żółtka jaja jest jednorodny przy badaniu za pomocą chromatografii kolumnowej, elektroforezy w wolnym roztworze, elektroforezy dyskowej

i immunoelektroforezy oraz podczas sedymentacji w ultrawirówce. Jest on izoelektryczny przy pH 4,16 i wykazuje ruchliwość elektroforetyczną przy pH 7,2 równą $15{,}76 \times 10^{-5}$ $cm^2.v^{-1}sek.^{-1}$.

2. Ciężar cząsteczkowy flawoproteidu oznaczony na podstawie sedymentacji, stałej dyfuzji i objętości właściwej wynosi 36 000.

3. Flawoproteid jest glikoproteidem zawierającym ok. 297 reszt aminokwasów, 21 reszt cukrowych oraz 1 resztę ryboflawiny na cząsteczkę białka. Flawoproteid jest prawdopodobnie dimerem złożonym z dwóch jednakowych łańcuchów polipeptydowych, posiadającym histidynę jako *N*-końcowy aminokwas.

4. Na podstawie różnic w ilości potencjometrycznie miareczkowanych grup pomiędzy flawo- i apoproteidem przedyskutowano wiązanie białka z flawiną.

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PREPARATION AND PROPERTIES OF SOME 4-SUBSTITUTED ANALOGUES OF CYTOSINE AND DIHYDROCYTOSINE

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1. The ready "leaving" tendency of the 4-ethoxy group in 4-ethoxy-2-ketopyrimidine and its methyl analogues has been profited from to prepare several 4-glycyl and 4-semicarbazido derivatives, some of the properties of which are described. 2. The 4-semicarbazido derivatives can be readily reduced on a rhodium catalyst; these reduced derivatives, in contrast to dihydrocytosine and other dihydropyrimidines, exhibit remarkable stability in alkaline medium. 3. The semicarbazido derivatives also readily undergo photohydration at the 5,6 bond, when irradiated at wavelengths to the red of 260 mμ; at shorter wavelengths they are radiation resistant. 4. The resulting photohydrates largely revert to the parent compounds in acid medium by elimination of the water molecule; but in alkaline medium they are remarkably stable. 5. In alkaline medium the 4-semicarbazido derivatives undergo some type of transformation, the nature of which has not been fully elucidated, but appears to involve saturation of the 5,6 bond.

During the course of a study on the nature of the products resulting from the reduction of cystosine and its glycosides in the presence of various amines (Janion & Shugar, 1967), including semicarbazide and amino acids such as glycine, as well as peptides, the necessity arose of preparing 4-glycyl-2-ketopyrimidine and 4-semicarbazido-2-ketopyrimidine.

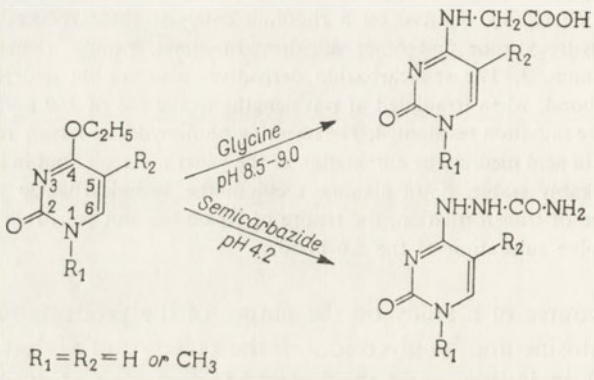
The preparation of such compounds as 4-glycyl-2-ketopyrimidine has been previously reported, the most recent method being that of Ueda & Fox (1963) based on the nucleophilic replacement of the thiol group of 4-thio-2-ketopyrimidine by an amino acid in slightly alkaline medium. The 4-semicarbazido derivative of cytidine has also been prepared by the direct reaction of cytidine with semicarbazide, which proceeds slowly in aqueous acid medium (Hayatsu & Ukita, 1964; Hayatsu, Takeishi & Ukita, 1966).

The present communication describes the synthesis of both of the foregoing compounds, as well as a number of their methylated and reduced derivatives, *via* 4-ethoxy-2-ketopyrimidine as the starting compound, and gives some of the properties of the products formed. These are of particular interest because of the potential applications of semicarbazide to the selective modification of cytosine residues in nucleic acids (Hayatsu & Ukita, 1966), especially tRNA; and the previous

demonstration of the possible existence of pyrimidynyl-4-amino acids in biological systems (Janion & Shugar, 1967). The spectral data are also of significance in studies on the properties of synthetic polynucleotides containing 4-semicarbazido-2-ketopyrimidine residues (Janion & Shugar, 1968).

RESULTS

The basis for the present procedure was the previous observation that 4-ethoxy-2-ketopyrimidine reacted very smoothly with hydroxylamine in aqueous medium to produce, initially, 4-hydroxylamino-2-ketopyrimidine (Janion & Shugar, 1965). The high "leaving tendency" of the 4-ethoxy group in this type of reaction, and the ease with which the reaction went to completion suggested its extension to the reaction with other amines. An additional factor of importance was the high solubility of the ethoxy derivatives with respect to the reaction products, which simplified the isolation of the latter. It was, in fact, found that the following two reactions proceed very smoothly in aqueous medium under the conditions described in Scheme 1:



Scheme 1

The difference in pH values required for appropriate reaction rates in each case is undoubtedly dependent on the extent of dissociation of the substituents (pK of semicarbazide 3.5; pK_2 of glycine 9.78), and not on that of 4-ethoxy-2-ketopyrimidine. The preparation of some of these compounds is described in detail under Experimental, below. The resulting products may be regarded as derivatives of cytosine in which one of the exo amino hydrogens has been replaced by an acetic acid or urea residue. The ultraviolet absorption spectra of the products are in accord with this and closely resemble those of cytosine itself.

Figures 1a and 1b exhibit the spectra of 4-glycyl-2-ketopyrimidine at various pH values. From the similarity of the curves in acid medium with those of cytosine, it is clear that the pK in this pH range is that for protonation of the ring $N_{(3)}$ nitrogen (Brooks & Lawley, 1962; Katritsky & Warring, 1962; Fikus, Wierzchowski & Shugar, 1962). The pK value, along with those for several other products discussed below,

are presented in Table 1. While the value for 4-glycyl-2-ketopyrimidine does not differ appreciably from that for 4-amino-2-ketopyrimidine (cytosine), it will be noted that substitution of the semicarbazido group in the 4-position leads to an appreciable decrease in the pK value.

Furthermore in alkaline medium the dissociation of 4-glycyl-2-ketopyrimidine is strikingly similar to that for cytosine itself (Shugar & Fox, 1952), with an estimated pK of about 12.6 (Fig. 1b), due to dissociation of the $N_{(1)}$ ring hydrogen. This value is to be compared with that for cytosine, about 12.4.

For the 4-semicarbazido derivatives the situation is quite analogous in acid medium, but departs appreciably from the expected behaviour in alkaline medium. The spectra of these compounds as a function of pH are illustrated in Figs. 2a, b and c. The resemblance of the spectral patterns, in acid medium, to those of the corresponding parent cytosine derivatives (Shugar & Fox, 1952; Fox & Shugar, 1952), again indicates unequivocally that they are the direct consequence of ring $N_{(3)}$ protonation, and the pK values estimated from the absorption spectra are listed in Table 1. It should also be noted that a comparison of the spectra at neutral and acid pH between 4-semicarbazido-2-ketopyrimidine (Fig. 2a) and 1-methyl-4-semicarbazido-2-ketopyrimidine (Fig. 2b), in the latter of which the 2-keto position is fixed, establishes the structure of the former in neutral medium; the observed similarity would not exist if the structure of the former were 4-semicarbazido-2-hydroxypyrimidine.

However, the absorption spectrum for 4-semicarbazido-2-ketopyrimidine in alkaline medium (Fig. 2a) is quite clearly not associated with ionization of the

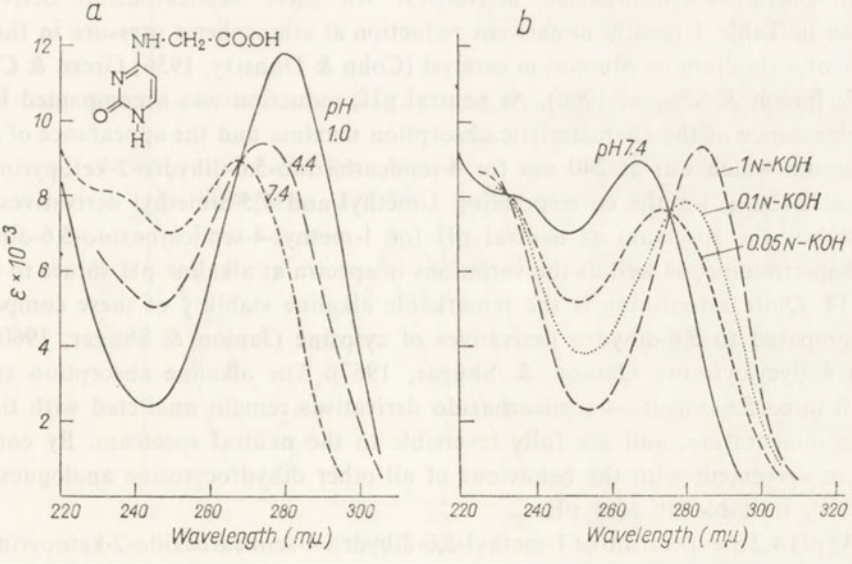


Fig. 1. Absorption spectrum of 4-glycyl-2-ketopyrimidine: (*a*), spectral modifications in acid medium associated with protonation of the ring $N_{(3)}$ (pK 4.4); (*b*), spectral modifications in alkaline medium associated with ionization of the ring $N_{(3)}$ hydrogen (pK ~ 12.6).

$N_{(1)}$ hydrogen, as is the case for 4-glycyl-2-ketopyrimidine (Fig. 1b) and cytosine (Shugar & Fox, 1952). If any doubts existed on this score, they are at once dispelled by a comparison with the alkaline absorption spectrum of 1-methyl-4-semicarbazido-2-ketopyrimidine (Fig. 2b) and 1,5-dimethyl-4-semicarbazido-2-ketopyrimidine (Fig. 2c), in both of which the $N_{(1)}$ position is effectively blocked. It follows that structural modifications accompanying alkalization, which are essentially similar for all three of the foregoing compounds, must be due to some modification of the semicarbazido moiety, or some interaction of the latter with the pyrimidine ring.

Table 1

pK values for protonation of ring $N_{(3)}$ *nitrogen in cytosine and several of its derivatives*

Compound	p*K*
Cytosine	4.45
4-Glycyl-2-ketopyrimidine	4.40
4-Semicarbazido-2-ketopyrimidine	3.20
1-Methyl-4-semicarbazido-2-ketopyrimidine	3.10
1,5-Dimethyl-4-semicarbazido-2-ketopyrimidine	3.60

Finally, attention should be drawn to the fact that, on prolonged exposure to alkali, the 4-semicarbazido derivatives undergo additonal transformations. These will be discussed below.

5,6-Dihydro-4-semicarbazido derivatives. All three semicarbazido derivatives shown in Table 1 readily underwent reduction at atmospheric pressure in the presence of a rhodium on aluminum catalyst (Cohn & Doherty, 1956; Green & Cohen, 1957; Janion & Shugar, 1960). At neutral pH, reduction was accompanied by the disappearance of the characteristic absorption maxima and the appearance of a new maximum which was at 240 mμ for 4-semicarbazido-5,6-dihydro-2-ketopyrimidine and at 242 mμ for the corresponding 1-methyl and 1,5-dimethyl derivatives. Fig. 3 exhibits the spectrum at neutral pH for 1-methyl-4-semicarbazido-5,6-dihydro-2-ketopyrimidine, as well as the variations in spectra at alkaline pH values to about pH 14. Quite astonishing is the remarkable alkaline stability of these compounds as compared to 5,6-dihydro derivatives of cytosine (Janion & Shugar, 1960) and even 4-glycylcytosine (Janion & Shugar, 1967). The alkaline absorption spectra of all three 5,6-dihydro-4-semicarbazido derivatives remain unaltered with time at room temperature, and are fully reversible to the neutral spectrum. By contrast, and in agreement with the behaviour of all other dihydrocytosine analogues, they are very unstable at acid pH.

At pH 4.2 the spectrum of 1-methyl-5,6-dihydro-4-semicarbazido-2-ketopyrimidine slowly disappears: at pH 3.5 this process is so rapid that it proved impossible to determine whether the ring nitrogen protonates as in the case of dihydrocytosine analogues. A chromatographic examination of the acid hydrolysis products, using

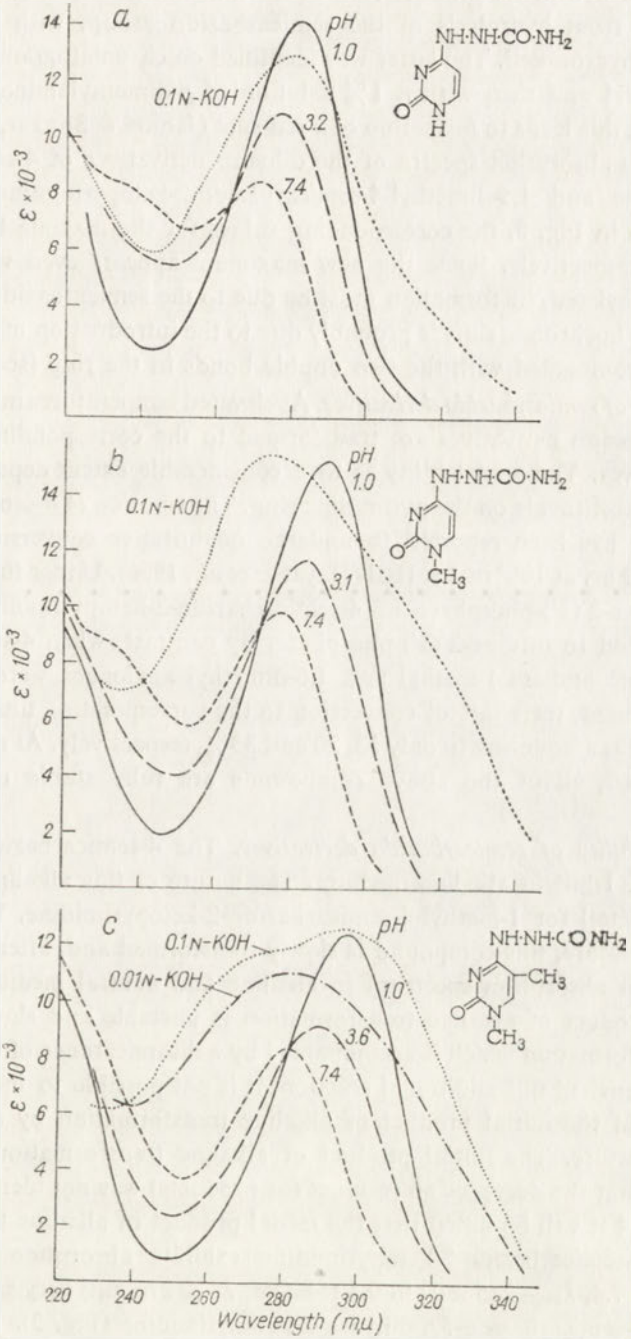


Fig. 2. Absorption spectra at various pH values of: (*a*), 4-semicarbazido-2-ketopyrimidine; (*b*), 1-methyl-4-semicarbazido-2-ketopyrimidine; (*c*), 1,5-dimethyl-4-semicarbazido-2-ketopyrimidine.

1-methyl-5,6-dihydrouracil as control, together with an examination of the alkaline spectrum of the hydrolysis products, indicated that the principal product in acid medium arises from hydrolysis of the semicarbazido group, with formation of 1-methyl-5,6-dihydrouracil. The latter was identified on chromatograms by spraying with 0.5 N-NaOH and then with a 1% solution of *p*-dimethylaminobenzaldehyde in acid ethanol; this leads to formation of a red spot (Janion & Shugar, 1960).

The alkaline absorption spectra of the dihydro derivatives of 4-semicarbazido-2-ketopyrimidine and 1,5-dimethyl-4-semicarbazido-2-ketopyrimidine are similar to those shown in Fig. 5, the corresponding values for the maxima being 263 mμ and 268 mμ, respectively. Since the new maximum appears even when the $N_{(1)}$ nitrogen is methylated, its formation must be due to the semicarbazido substituent; such a large bathochromic shift is probably due to the introduction of an additional double bond, conjugated with the two double bonds in the ring (see Discussion).

Acid lability of semicarbazido derivatives. At elevated temperatures in acid medium, the 4-semicarbazido derivatives are transformed to the corresponding 4-keto, i.e. uracil, derivatives. The acid lability is to a considerable extent dependent on the nature of the substituents on the pyrimidine ring. The riboside of 4-semicarbazido-2-ketopyrimidine has been reported to undergo quantitative conversion to uridine when kept for 2 hr. at 100° in 1 N-HCl (Hayatsu *et al.*, 1966). Under the same conditions the ribose-2′(3′)-phosphate of 4-semicarbazido-2-ketopyrimidine is quantitatively converted to uridine-2′(3′)-phosphate. By contrast, when 4-semicarbazido-2-ketopyrimidine and its 1-methyl and 1,5-dimethyl analogues were subjected to the same treatment, their rate of conversion to the corresponding uracil derivatives was much slower, amounting to only 33, 20 and 33%, respectively. At room temperature in 1 N-HCl, all of the above compounds are fully stable over a period of 24 hr.

Alkaline lability of semicarbazido derivatives. The 4-semicarbazido derivatives are much more labile in alkaline medium. The nature of this alkaline lability was examined in detail for 1-methyl-4-semicarbazido-2-ketopyrimidine. In 0.1 N-KOH at room temperature, this compound is slowly transformed and, after about 24 - 48 hr., exhibits an absorption spectrum in alkaline and neutral media as shown in Fig. 4. The product of alkaline transformation is unstable and slowly undergoes a further transformation which is accompanied by a disappearance of the absorption spectrum. Because of this additional reaction, it is not possible to increase the rate of formation of the initial product of alkaline transformation by an increase in pH or temperature. The initial product of alkaline transformation is extremely labile in acid, but the degradation product (or products) was not identified.

From Fig. 4 it will be noted that the initial product of alkaline transformation of 1-methyl-4-semicarbazido-2-ketopyrimidine exhibits absorption maxima and spectra (at pH 7.4, λ_{max} 245 mμ; in 0.1 N-KOH, λ_{max} 270 mμ) very similar to those for 1-methyl-4-semicarbazido-5,6-dihydro-2-ketopyrimidine (Fig. 3). This similarity in absorption spectra suggested that the transformation reaction in alkaline medium involved some modification of the 5,6 bond, leading to its saturation. If this were so, then the modification in absorption spectrum in going from neutral to alkaline

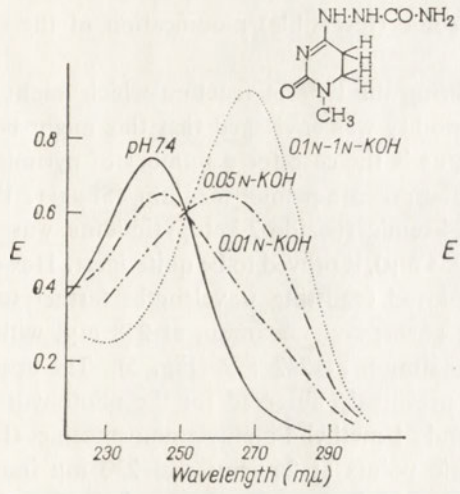


Fig. 3

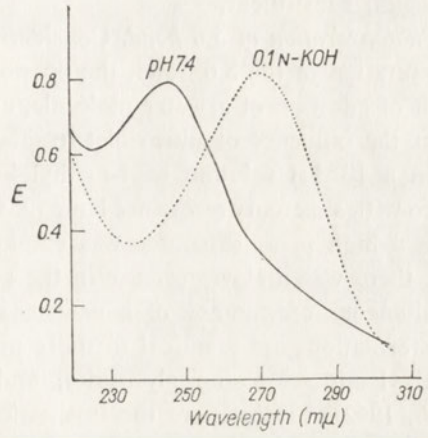


Fig. 4

Fig. 3. Absorption spectrum at neutral and alkaline pH of the 5,6-dihydro derivative of 1-methyl-4-semicarbazido-2-ketopyrimidine.

Fig. 4. Absorption spectrum of the alkaline transformation product of 1-methyl-4-semicarbazido-2-ketopyrimidine (10^{-4} M) after 48 hr. in 0.1 N-KOH at room temperature, and measured in 0.1 N-KOH. Neutralization of this solution gave spectrum at pH 7.4.

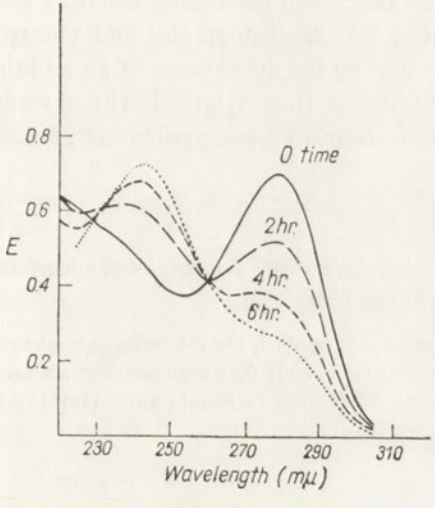


Fig. 5

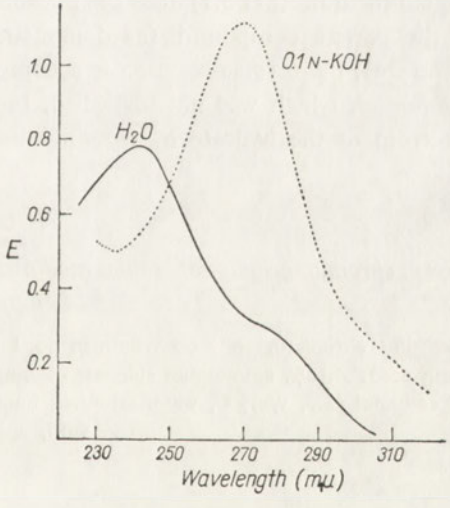


Fig. 6

Fig. 5. Course of photochemical hydration of 1-methyl-4-semicarbazido-2-ketopyrimidine. A 10^{-4} M aqueous solution was irradiated directly in a 10-mm. spectrophotometer cuvette with a Q-700 mercury lamp, from which radiation below 230 mμ was cut off by means of a 5-mm. layer of saturated ammonium acetate.

Fig. 6. Absorption spectrum of the hydrate of 1-methyl-4-semicarbazido-2-ketopyrimidine in water (pH ~ 6) and in 0.1 N-KOH. The shoulders in both spectra at long wavelengths are due to the presence of some of the non-reacted parent compound.

pH (Fig. 4) must necessarily involve some (reversible) modification of the semicarbazido substituent.

Photohydration of 5,6 bond. Considering the type of reaction which might lead to saturation of the 5,6 bond, the possibility was envisaged that this might be the result of addition of a water molecule, as is the case for a number of pyrimidines under the influence of ultraviolet irradiation in aqueous medium (Shugar, 1960). When a 10^{-4} M solution of 1-methyl-4-semicarbazido-2-ketopyrimidine was irradiated with a mercury resonance lamp (254 mμ), it proved to be quite inert. However, when a high pressure source was employed (emitting wavelengths farther to the red), there was a slow decrease in the absorption maximum at 278 mμ, with the simultaneous appearance of a new maximum at 242 mμ (Fig. 5). The spectral transformations are identical to those previously observed for the photohydration of alkylamino cytosine glycosides, and 1-methyl-4-methylaminocytosine (Fikus *et al.*, 1962). Furthermore the isosbestic points at 260 mμ and 230 mμ indicate the formation of only one photoproduct. Note from the figure that after 6 hr. irradiation the reaction has still not gone to completion (absorption at 278 mμ).

It was previously shown that the hydrate of 1-methyl-4-methylamino-2-ketopyrimidine (Fikus *et al.*, 1962) is much more stable than the hydrate of 1-methylcytosine. However, the stability of the hydrate of 1-methyl-4-semicarbazido-2-ketopyrimidine far transcends those of the foregoing hydrates; in neutral medium or in 0.1 N-KOH it is fully stable at room temperature for more than 24 hr. Only in acid medium (pH 3.1) does the photohydrate exhibit instability, reverting largely to the parent compound (as demonstrated by chromatography and absorption spectrum); the reverse reaction is accompanied by the appearance of an additional compound, which was not identified, but was not 1-methyluracil. The absorption spectrum of the hydrate of 1-methyl-4-semicarbazido-2-ketopyrimidine at neutral

Table 2

Paper chromatography of some modified derivatives of 1-methyl-4-semicarbazido-2-ketopyrimidine

Ascending chromatography on Whatman no. 1 paper was applied, the following solvent systems being used: *A*, 1% ammonium sulphate - isopropanol (1:2, v/v); *B*, 1 M-ammonium acetate, pH 7.4 - ethanol (2:5, v/v); *C*, water-saturated butanol; *D*, benzene - ethanol - water (169:45:15, by vol.), upper phase.

Compound	R_F in solvent			
	A	*B*	*C*	*D*
1-Methyl-4-semicarbazido-2-ketopyrimidine	0.68	0.72	0.10	0.00
1-Methyl-4-semicarbazido-2-ketopyrimidine·H_2O	0.86	0.90	0.22	0.00
1-Methyl-4-semicarbazido-2-ketopyrimidine·H_2	0.86	0.90	0.31	0.13
Transformation product				
in unbuffered alkali, pH 11 - 13	0.86	0.90	0.22	0.00
in borate buffer, 0.2 M, pH 10	—	0.00	0.00	—
in glycine buffer, 0.2 M, pH 10	—	0.00	0.00	—

and alkaline pH values is presented in Fig. 6. A comparison with Fig. 4 shows that both the location of the maximum and the shape of the spectrum differ from the product formed in alkaline medium without irradiation. Taken in conjunction with the different behaviour of the two products in acid, it is clear that the product formed in alkaline medium is different from that formed by irradiation.

Influence of buffer on product formed in alkaline medium. Since the semicarbazido group in 1-methyl-4-semicarbazido-2-ketopyrimidine is almost entirely in the ionized form in 0.1 N-KOH (p*K* about 12.2), the question arose as to whether there is any relationship between the dissociation of the semicarbazido group and the alkaline instability of the product. It was, in fact, found that the rate of alkaline transformation of 1-methyl-4-semicarbazido-2-ketopyrimidine is almost identical at pH 10, 12 and 13. The reaction takes place even at pH 9, but at a considerably lower rate.

It was observed, during the course of these experiments, that at pH 10, where buffering is required to maintain the pH, the products formed in the presence of various buffers are not identical. Small, but significant differences were found between the products formed in the presence of borate or glycine buffers, or in KOH alone. These differences are also reflected in the R_F values of the products, which are shown in Table 2. Only neutral solvent systems were employed in these experiments because of the lability of the products.

EXPERIMENTAL

4-Ethoxyuracil and its methylated derivatives were obtained by the methods of Hilbert & Jansen (1935) and Hilbert & Johnson (1930). Reactions were followed by paper chromatography, using Whatman no. 1 and water-saturated butanol with ammonia in the vapour phase. R_F values for the various compounds were as follows: 4-ethoxyuracil, 0.76; 4-glycyl-2-ketopyrimidine, 0.00; 4-semicarbazido-2-ketopyrimidine, 0.00; 1-methyl-4-ethoxy-2-ketopyrimidine, 0.85; 1-methyl-4-semicarbazido-2-ketopyrimidine, 0.08; 1,5-dimethyl-4-ethoxy-2-ketopyrimidine, 0.88; 1,5-dimethyl-4-semicarbazido-2-ketopyrimidine, 0.20.

4-Glycyl-2-ketopyrimidine (*4-glycyluracil*). With the exception that the starting product was different, the method employed was similar to that of Ueda & Fox (1963). 27.5 mg. 4-ethoxyuracil (0.196 m-moles) was dissolved with heating in 0.6 ml. 2.5 M-glycine (1.5 m-moles) in a small test-tube. The solution was brought to pH 8.5 - 9.0 by addition of about 12 mg. sodium carbonate, the tube sealed and then brought to 80° for 2 hr. Chromatography at this point showed that the reaction had gone to completion; but only when the above reaction conditions were strictly adhered to. If the concentration of glycine, or the pH of the solution, are lower than those indicated above, the final products include some uracil.

The reaction mixture was brought to room temperature, acidified with formic acid to precipitate the glycyluracil, and the latter collected by centrifugation and washed with diluted formic acid to obtain 31 mg. product (94% yield). The crystals

were dissolved in dilute ammonia and recrystallized by acidification with formic acid. The product does not exhibit a defined melting point.

Spectral data: (a) pH 7.4, λ_{max} 268 mμ, ε_{max} 8.9×10^3; λ_{min} 248 mμ, ε_{min} 7.0×10^3; inflexion at 235 mμ with $\varepsilon = 8.1\times10^3$; (b) in 0.1 N-HCl, λ_{max} 280 mμ, ε_{max} 11.8×10^3; λ_{min} 242 mμ, ε_{min} 2.4×10^3; (c) in 0.1 N-KOH, λ_{max} 285 mμ, ε_{max} 9.34×10^3; λ_{min} 255 mμ, ε_{min} 2.3×10^3.

Note. Under the above conditions, 4-ethoxyuracil reacts readily with all amino acids or peptides to give the corresponding 4-aminoacid-2-ketopyrimidines or 4-peptidyl-2-ketopyrimidine.

Preparation of 4-semicarbazido derivatives of 2-ketopyrimidine

The riboside of 4-semicarbazido-2-ketopyrimidine was first obtained by Hayatsu & Ukita (1964) by treating cytidine at pH 4.2 and 37° with a large excess of semicarbazide. The procedure employed here was similar with the exception that the starting compound was a 4-ethoxypyrimidine derivative.

The appropriate 4-ethoxy derivative was dissolved in 2.5 M-semicarbazide at pH 4.2, the ratio of the latter to the former being at least 2, and the reaction mixture kept at 37°. The course of the reaction was followed by paper chromatography until the 4-ethoxy derivative had been completely transformed. Due to its lower solubility, the reaction product crystallized out spontaneously. When the reaction was complete, the crystals were collected, washed and recrystallized from water.

1-Methyl-4-semicarbazido-2-ketopyrimidine. 22.4 mg. of 1-methyl-4-ethoxypyrimidine was dissolved in 0.25 ml. of 2.5 M-semicarbazide hydrochloride which had previously been brought to pH 4.2 by addition of 50 μl. 10 N-NaOH. The reaction went to completion in 2 days at 37°. The resulting crystalline precipitate was washed with water, ethanol and dried to give 25.4 mg. product, yield 96%. Following recrystallization from water, m.p. 172 - 173°. Spectral data: (a) pH 7.4, λ_{max} 278 mμ, ε_{max} 9.7×10^3; λ_{min} 235 mμ, ε_{min} 5.6×10^3; (b) in 0.1 N-HCl, λ_{max} 290 mμ, ε_{max} 15.1×10^3; λ_{min} 245 mμ, ε_{min} 1.9×10^3; (c) in 0.1 N-KOH, λ_{max} 275 mμ, ε_{max} 15.5×10^3; λ_{min} 230 mμ, ε_{min} 7.4×10^3.

4-Semicarbazido-2-ketopyrimidine. 10 mg. of 2-keto-4-ethoxypyrimidine was dissolved by warming in 0.25 ml. of 2 .5 M-semicarbazide at pH 4.2. After 24 hr. the crystals formed were removed and an additional 10 mg. 2-keto-4-ethoxypyrimidine added. Following an additional 24 hr., a further 9 mg. was added to the supernatant. The final yield of crystalline product, following washing with water and alcohol, was 27.5 mg. (yield 82%). The product was recrystallized from water, but had no defined melting point. Spectral data: (a) pH 7.4, λ_{max} 272 mμ, ε_{max} 8.4×10^3; λ_{min} 252 mμ, ε_{min} 6.7×10^3; (b) in 0.1 N-HCl, λ_{max} 282 mμ, ε_{max} 14.1×10^3; λ_{min} 242 mμ, ε_{min} 2.8×10^3; (c) in 0.1 N-KOH, λ_{max} 280 mμ, ε_{max} 12.93×10^3; λ_{min} 243 mμ, ε_{min} 5.83×10^3.

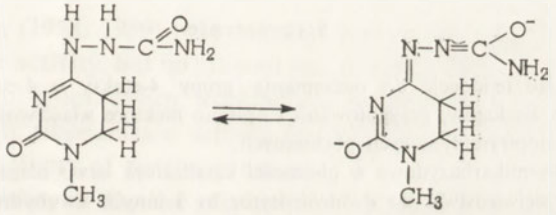
1,5-Dimethyl-4-semicarbazido-2-ketopyrimidine. 21 mg. 1,5-dimethyl-4-ethoxyuracil was dissolved with heating in 100 μl. of 2.5 M-semicarbazide at pH 4.2. The reaction

went to completion in 4 days, following which the crystalline precipitate was collected and dried to give 16.7 mg. (yield 68%). The lower yield in this instance was due simply to the increased solubility of the product. Recrystallization proceeded with the same yield to give a product with m.p. <360°. Spectral data: (a) pH 7.4, λ_{max} 281 mμ, ε_{max} 8.35×10^3; λ_{min} 253 mμ, ε_{min} 3.6×10^3; (b) in 0.1 N-HCl, λ_{max} 296 mμ, ε_{max} 12.5×10^3; λ_{min} 252 mμ, ε_{min} 860; (c) in 0.1 N-KOH, λ_{max} 295 mμ, ε_{max} 12.6×10^3; λ_{min} 232 mμ, ε_{min} 6.1×10^3; inflexion at 285 mμ, with $\varepsilon = 12.06\times10^3$.

DISCUSSION

Two points require further elucidation, viz. the nature of the reversible dissociation of the 5,6-dihydro-4-semicarbazido derivatives in alkaline medium, and the mechanism of the alkaline transformation of 1-methyl-4-semicarbazido-2-ketopyrimidine.

As regards the dissociation of the 4-semicarbazido derivatives in alkaline medium, it is most convenient to consider the case of the 1-methyl-5,6-dihydroderivative, in which the ring $N_{(1)}$ is blocked. This compound, as well as the other two related ones, possesses two conjugated bonds in neutral medium. The marked bathochromic shift and increase in extinction in alkaline medium (Fig. 3) can be readily interpreted on the basis of the appearance of a third conjugated double bond, as follows:



This is most likely the path for alkaline dissociation not only of the other 5,6-dihydro-4-semicarbazido analogues, but also of the remaining 4-semicarbazido derivatives.

As concerns the alkaline transformation product of 1-methyl-4-semicarbazido-2-ketopyrimidine on prolonged exposure to alkali, the relative instability of the resulting product renders positive identification rather difficult in the absence of additional suitable comparison analogues. However, the striking similarity of the spectrum of the alkaline transformation product with those for the corresponding reduced and photohydrated products implies some type of saturation of the 5,6 bond.

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PREPARATYKA I WŁAŚCIWOŚCI NIEKTÓRYCH ANALOGÓW CYTOZYNY I DWUHYDROCYTOZYNY PODSTAWIONYCH PRZY $C_{(4)}$

Streszczenie

1. Wykorzystując tendencje do odczepiania grupy 4-etoksy u 4-etoksy-2-ketopirymidyny i jej metylowanych analogów, przygotowano i opisano niektóre właściwości kilku 4-glicyno i 4-semikarbazydo-2-ketopirymidynowych pochodnych.

2. Pochodne 4-semikarbazydowe w obecności katalizatora łatwo ulegają redukcji, a powstające związki w przeciwieństwie do dwuhydrocytozyny i innych dwuhydropirymidyn są bardzo trwałe w środowisku alkalicznym.

3. Pochodne semikarbazydowe naświetlane długością fali powyżej 260 mμ ulegają również reakcji uwodnienia w obrębie wiązania 5,6; naświetlanie krótszymi falami nie powoduje żadnych zmian.

4. Powstały fotoprodukt w warunkach kwaśnych traci wodę i w znacznym procencie wytwarza związek macierzysty, natomiast w środowisku alkalicznym jest bardzo trwały.

5. Pochodne 4-semikarbazydowe w środowisku alkalicznym ulegają pewnym, jeszcze niedokładnie określonym przemianom, które najprawdopodobniej polegają na nasyceniu wiązania 5,6.

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RIBONUCLEATE 3′-NUCLEOTIDOHYDROLASE FROM PIG PITUITARY GLAND

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1. Ribonuclease preparation from pig pituitary gland purified 45-fold was free of phosphatase, nucleotidase, DNase and phosphodiesterase activities. 2. The enzyme did not require mono- or divalent cation for activity, and its pH optimum was 6.5. 3. The ribonuclease acted on yeast RNA as an exonuclease, liberating 3′-nucleotides. No cyclic nucleotides were found in the digest, and cytidine 2′:3′-cyclic phosphate was not hydrolysed. 4. The molecular weight of the enzyme was estimated to be 104 000.

La Bella & Brown (1958, 1959) reported that bovine and pig pituitary glands contained ribonuclease activity, but no attempt was undertaken to isolate the enzyme and investigate its properties. When working on enzymes of pig pituitary gland we have found that its ribonuclease activity (calculated per gram of tissue) was twice as high as the activity of bovine pancreas.

To study the possibility of the use of pituitary RNase for the determination of polynucleotide sequence, the enzyme was partially purified and its properties and specificity were investigated.

MATERIAL AND METHODS

Chemicals. The following reagents were used: Adenosine 3′-phosphate, uridine 2′- and 3′-phosphate (mixed isomers) (T. Schuchardt, München, West Germany), cytidine 3′-phosphate (Biuro Obrotu Odczynnikami Chemicznymi, Gliwice, Poland), guanosine 2′- and 3′-phosphate (mixed isomers) (Koch-Light, Colnbrook, Bucks, England), adenosine 5′-phosphate and sodium nucleinate from yeast (British Drug Houses, Poole, England), uridine 5′-phosphate, guanosine 5′-phosphate, cytidine 2′:3′-cyclic phosphate and bovine pancreatic ribonuclease (Sigma, St. Louis, Mo., U.S.A.), bis-*p*-nitrophenylphosphate (Calbiochem., Los Angeles, Calif., U.S.A.), DEAE-cellulose De-50 (W. & R. Balston, England), CM-Sephadex C-50 and Sephadex G-200 (Pharmacia, Uppsala, Sweden). DNA from rat liver was prepared by the method of Kirby (1957).

Standards for molecular weight determination. Pepsin (mol. wt. 35 700) and trypsin (mol. wt. 23 800) were products of T. Schuchardt (München, West Germany); glyceraldehydephosphate dehydrogenase from human muscle (mol. wt. 125 000) was prepared according to Wolny (1968), and mucoid from horse erythrocytes (mol. wt. about 10^6) was obtained by the method of Lisowska (1962).

Enzymes assays. RNase was determined by the modified Kunitz method as described by McDonald (1955a). Commercial RNA was dialysed successively against 1 M-NaCl and water, and freeze-dried. For the assay, 1 ml. of 0.6% sodium nucleinate in 0.1 mM-EDTA - 0.1 M-tris-HCl buffer, pH 7.4, and 1 ml. of the enzyme solution in the same buffer were incubated for 10 min. at 25°, or for 30 min. at 37°. The test with shorter time and lower temperature was used during the purification procedure, that with longer time and at 37° was used for the determination of the properties of the enzyme. The reaction was stopped by the addition of 2 ml. of 0.4% uranyl acetate in 4% trichloroacetic acid. After 10 min. the mixture was filtered through Whatman no. 1 paper, or centrifuged for 2 min. at 4000 rev./min., and in the supernatant the RNA digestion products were measured by the determination of total phosphorus (Fiske & Subbarow, 1925) or spectrophotometrically at 260 mμ.

A unit of RNase activity was defined as that amount of the enzyme which under the described conditions gave an increase in extinction at 260 mμ equal to 1.0.

When cytidine 2′:3′-cyclic phosphate was used as substrate, 2 ml. of 15 mM solution was incubated at pH 7.5 with 0.5 ml. of 0.33 mM-EDTA and enzyme, the total volume being adjusted with 0.1 M-NaCl to 10 ml., and the activity determined with a pH-stat at 20° for 20 min. (Bergmeyer, 1962).

DNase activity was measured by the method of McDonald (1955b) with a 0.3% solution of rat-liver DNA in 0.1 M-succinic acid-NaOH buffer, pH 5.8, containing 3 mM-EDTA. The activity was determined in the presence and in the absence of Mg^{2+} ion.

Phosphatase activity was determined by the method of King & Armstrong (King, 1952) at pH 5.8 and 8.8 using phenylphosphate as substrate in the presence and in the absence of Mg^{2+} ion.

Nucleotidase activity was measured by the method of Martin & Doty (1949) with 5 mM-adenosine 3′-phosphate and adenosine 5′-phosphate in 0.2 M-glycine-NaOH buffer, pH 8.8.

Phosphodiesterase activity was assayed by the method of Privat de Garilhe & Laskowski (1955) with 2 mM-bis-*p*-nitrophenylphosphate in 0.3 M-tris-HCl buffer, pH 8.6 and in 0.3 M-tris-maleinate buffer, pH 6.7, in the presence and in the absence of Mg^{2+} ion.

Analytical methods. Starch-gel electrophoresis was carried out according to the procedure of Smithies (1959).

Molecular weight estimation was made by means of Sephadex G-200 gel filtration according to Wieland, Duesberg & Determan (1963). A column (1.5×105 cm.) equilibrated with 0.1 M-phosphate buffer containing 0.1 mM-EDTA, pH 6.3,

was used. Pepsin, trypsin, glyceraldehydephosphate dehydrogenase and mucoid were used as standards.

The products of partial and total enzymic digestion of yeast RNA were analysed by paper chromatography and high-voltage electrophoresis. The paper chromatography was performed on Whatman no. 3 *MM* paper using isopropanol - water (70:30, v/v) and ammonia in vapour phase (Markham & Smith, 1952a, b); the paper was prewashed successively with 1 N-HCOOH, water, 0.25% sodium versenate, pH 9.5, and water (Baranowski & Długajczyk, 1959). For the paper electrophoresis a system of pyridine - acetic acid - water (1:10:89, by vol.), pH 3.5, described by Ryle, Sanger, Smith & Kitai (1955) was used. High-voltage electrophoresis was carried out for 75 min. at pH 3.5 and 6.5 at a voltage of 61 v/cm., and at pH 5.0 at 26 v/cm. (Markham & Smith, 1952b; Smith, 1955).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) or spectrophotometrically at 280 mμ.

RESULTS

Enzyme preparation. All steps of purification were carried out at 4°. For isolation of RNase whole pig pituitary glands were used, although the anterior lobe contains 8 times as much enzyme activity as the posterior lobe. The course of purification is presented in Table 1.

Table 1

Purification of pig pituitary ribonuclease

For purification, 126 g. of pig pituitary glands were taken. A unit of RNase is the amount of the enzyme which gives an increase in extinction at 260 mμ equal to 1.0.

Purification step	Volume (ml.)	Protein content (mg.)	Activity	
			units	units/mg. of protein
Initial 0.1 M-$(NH_4)_2SO_4$ extract	630	9380	2432	0.26
Ppt. at 30 - 60% ammonium sulphate sat.	115	2570	1787	0.7
DEAE-cellulose:				
fraction α	718	716	1434	2.0
fraction β	445	123	130	1.0
CM-Sephadex:				
fraction α'	48	323	453	1.4
fraction α''	63	54	637	11.8

Fresh, frozen pig pituitary glands, 126 g., obtained from the slaughter-house were ground in a tissue disintegrator and treated with 630 ml. of cold (4°) 0.1 M-ammonium sulphate solution. pH of the mixture was adjusted to 7.8 with 0.5 M-NaOH, a few drops of toluene were added and the suspension was mechanically stirred for

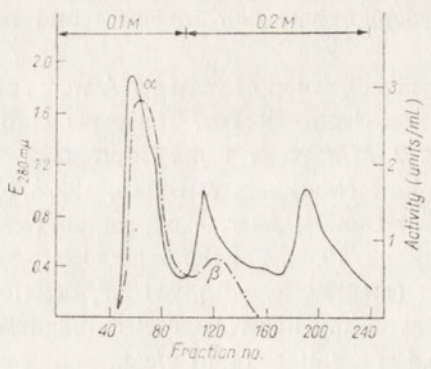

Fig. 1

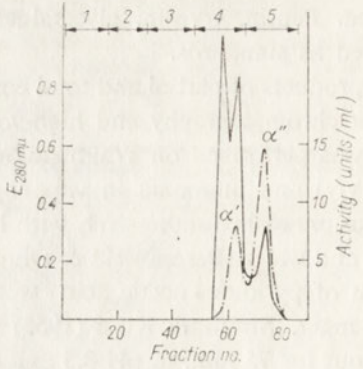

Fig. 2

Fig. 1. Chromatography on DEAE-cellulose of the pituitary RNase preparation. A column (3.4 × 66 cm.) was equilibrated with 0.01 M-sodium phosphate - 0.1 mM-EDTA buffer, pH 6.3. To the column 40 ml. of a solution of the 30 - 60% ammonium sulphate sat. precipitate (684 mg. of protein), pH 6.95, was applied, and eluted with a stepwise gradient of sodium phosphate buffer containing 0.1 M-EDTA, pH 6.3: (*1*), 450 ml. of 0.1 M-buffer; (*2*), 600 ml. of 0.2 M-buffer. The flow rate was 24 ml./hr. Fractions of 6 ml. were collected and in every second tube (—·—), the enzymic activity and (—), protein content were estimated.

Fig. 2. Chromatography on CM-Sephadex of fraction α from DEAE-cellulose column. To the column (1.6 × 20 cm.) equilibrated with 17 mM-sodium succinate - 0.1 mM-EDTA buffer, 18 ml. of the enzyme preparation (23 mg. of protein) was applied. For the elution a stepwise gradient of sodium succinate - 0.1 mM-EDTA, pH 6.3, was used, 50 ml. of each eluent being applied. (*1*), 1.7 mM-buffer; (*2*), 6.7 mM-buffer; (*3*), 17 mM-buffer; (*4*), 17 mM-buffer containing 0.2 M-NaCl; (*5*), 17 mM-buffer containing 0.5 M-NaCl. The flow rate was 12 ml./hr. Fractions of 3 ml. were collected and in every second tube (—·—), the enzymic activity and (—), the protein content were estimated.

20 hr. Then the precipitate was removed by centrifugation for 15 min. at 3000 rev./min., and to the supernatant (630 ml.) 107 g. of solid ammonium sulphate was added (30% saturation); pH was adjusted to 6.15 with 0.5 N-H_2SO_4. After 15 min. the precipitate was centrifuged off at 14 000 rev./min. (10 min.), and the supernatant was brought to 60% saturation by adding 118 g. of ammonium sulphate. pH was adjusted to 6.15 with 0.5 N-H_2SO_4 or NaOH, and after 15 min. the suspension was centrifuged as before. The precipitate was dissolved in 105 ml. of 0.01 M-sodium phosphate - 0.1 mM-EDTA buffer, pH 6.3, and dialysed against two changes of 2 litres of the same buffer. The dialysed solution was applied to the DEAE-cellulose column and two active fractions were obtained (Fig. 1). The first fraction representing 80% of the activity applied to the column was used for further purification. After dialysis against 1.7 mM-succinic acid-NaOH buffer containing 1 mM-EDTA, pH 6.3, the fraction was applied to CM-Sephadex column and again two active fractions were obtained (Fig. 2). The recovery of the applied activity was 77%, the first peak α′ containing 32% and the second α′′ 45%. The fraction α′′ which contained 26% of the starting activity was purified 45-fold. On starch-gel electrophoresis at pH 8.5 and 6.7, it separated into two fractions, both moving

towards the cathode (Fig. 3). Despite its non-homogeneity, the whole fraction α'' was used for further study.

Properties of the purified RNase (the α'' fraction). The molecular weight of fraction α'' estimated by gel filtration on Sephadex G-200 was 104 000 (Fig. 4).

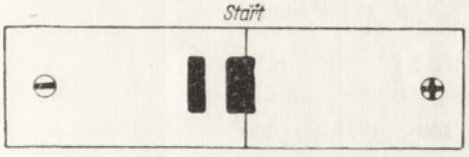

Fig. 3

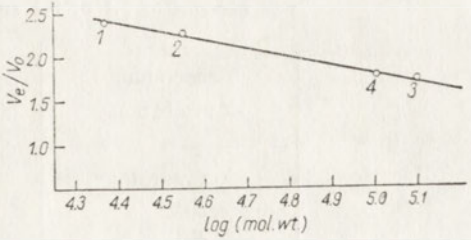

Fig. 4

Fig. 3. Starch-gel electrophoresis of the purified pig pituitary RNase (fraction α''). Conditions: 0.04 M-phosphate buffer, pH 6.7, 9.6 v/cm., 5 hr.; 0.05 ml. of 1% aqueous solution of enzyme was applied.

Fig. 4. Determination of molecular weight of ribonuclease on Sephadex G-200 column. The method of Wieland *et al.* (1963) was used. (*1*), Trypsin; (*2*), pepsin; (*3*), glyceraldehydephosphate dehydrogenase; (*4*), ribonuclease from pituitary gland.

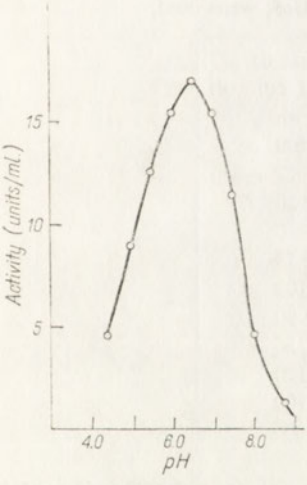

Fig. 5

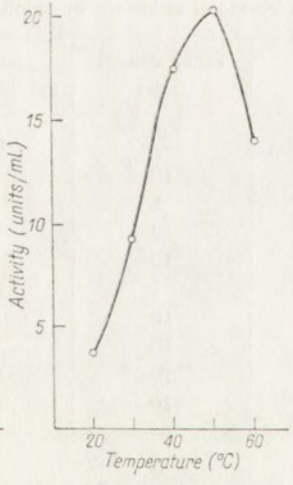

Fig. 6

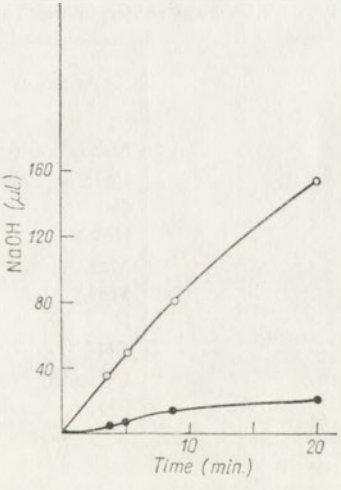

Fig. 7

Fig. 5. The effect of pH on the activity of the pituitary RNase. The buffer mixture contained: 0.02 M-tris-ammonium acetate - 0.02 M-KH_2PO_4 (Beard & Razzell, 1964). The incubation mixture was adjusted to the required pH with acetic acid or ammonia.

Fig. 6. The effect of temperature on the activity of the pituitary RNase. The activity was measured in 0.1 M-tris-HCl buffer, pH 7.4.

Fig. 7. Hydrolysis of cytidine 2′:3′-cyclic phosphate by (○), pancreatic and (●), pituitary RNases. The activity was measured according to Bergmeyer (1962). The reaction mixture contained: 2 ml. of 15 mM-cytidine 2′:3′-cyclic phosphate, 0.5 ml. of 0.33 M-EDTA, 6.5 ml. of 0.1 M-NaCl, and 1 ml. of enzyme solution containing 100 μg. of crystalline pancreatic RNase or 600 μg. of pituitary RNase (fraction α'').

Table 2

The effect of temperature on the stability of pituitary RNase at two pH *values*

The enzyme was incubated at pH 5.1 and 8.1 for 5 min. at the temperature indicated, then brought to 37° and the activity was assayed at pH 7.4 and 37°. In the controls, the pH of the enzyme solution was brought to 5.1 or 8.1 and immediately adjusted to 7.4.

Temperature of incubation	Activity (% of control)	
	pH 5.1	pH 8.1
Control	100	100
20°	95	80
40°	106	82
60°	100	19
80°	11	0

Table 3

Effect of various ions and cysteine on the activity of pituitary RNase

The incubation mixture contained in a final volume of 1 ml.: 3 mg. of sodium nucleinate, 0.1 m-mole tris-HCl buffer, 0.1 μmole EDTA and metal salts or other reagents at final concentration as shown in the Table. Metal chlorides or sulphates were used.

Addition	Final concn. (mM)	Activity (% of control)
None, control	—	100
Ca^{2+}	10	27
Cu^{2+}	1	20
Hg^{2+}	10	0
Mg^{2+}	10	73
Mn^{2+}	1	102
F^-	10	90
CN^-	10	26
Cysteine	10	100
EDTA	10	100

The enzyme was found to have a sharp pH optimum at 6.5 (Fig. 5) and in 0.1 M-tris-HCl buffer, pH 7.4, the optimum temperature was 50° (Fig. 6). The effect of temperature on the stability of the enzyme at two different pH values is presented in Table 2. The stability at pH 5.1 was higher than at pH 8.1. When the enzyme was incubated for 1 hr. at 4° and pH 2.2, or 11.2, the activity was completely lost.

The effect of various ions and cysteine is shown in Table 3. The enzyme was completely inactivated by Hg^{2+} ion at 10 mM concn. and only 20% of the original activity was observed in the presence of Cu^{2+}, Ca^{2+} and CN^- ions. EDTA up to 10 mM concentration and cysteine had no effect.

Table 4

Effect of nucleotides on the activity of pituitary RNase

Addition	Final concn. (mM)	Activity (% of control)
None, control	—	100
Guanosine 2′- and 3′-phosphate (mixed isomers)	0.3	83
Adenosine 3′-phosphate	0.17	98
Cytidine 3′-phosphate	0.3	96
Uridine 2′- and 3′-phosphate (mixed isomers)	0.3	63

Table 5

Effect of tris and NaCl *concentration on the activity of pituitary RNase*

The incubation was carried out at pH 7.4.

Buffer concn. (M)	NaCl concn. (M)	Activity (% of control)
Tris-HCl, 0.1 (control)	—	100
Tris-HCl, 0.025	—	69
Tris-HCl, 0.05	—	74
Tris-HCl, 0.05	NaCl, 0.1	64
Tris-HCl, 0.05	NaCl, 0.2	44
Tris-HCl, 0.05	NaCl, 0.5	32
Tris-HCl, 0.05	NaCl, 0.75	25

Among the nucleotides tested (Table 4) only the mixture of uridine 2′ and 3′-phosphate inhibited by 40% the enzymic reaction; other nucleotides were without any effect.

Pituitary RNase, similarly as the pancreatic RNase, was inhibited by high concentrations of NaCl (Table 5).

The purified enzyme showed no activity toward DNA, phenylphosphate, adenosine 3′-phosphate, adenosine 5′-phosphate or bis-*p*-nitrophenyl phosphate, and either with cytidine 2′:3′-cyclic phosphate as substrate no activity was observed (Fig. 7).

Products of digestion of yeast RNA by pituitary RNase. For determination of enzymic specificity, a partial and a limit digest of yeast RNA were investigated. To obtain a partial digest, 2.4 mg. of yeast RNA and 2680 units of the purified enzyme preparation were incubated at 37° for 3 hr. with the addition of a few drops of toluene. A control containing no enzyme was incubated simultaneously. To obtain the limit digest, 0.8 mg. of yeast RNA and 5300 units of the enzyme were incubated under the same conditions. After incubation the unhydrolysed RNA was

precipitated with 0.5% uranyl acetate in 5% trichloroacetic acid and its content estimated by the method of Mejbaum (1939). During the partial digestion 20% of RNA was hydrolysed and during the limit digestion 77% of RNA. In the supernatant the digestion products were analysed by paper chromatography (samples of 0.05 ml.) and electrophoresis at pH 3.5 (samples of 0.15 ml.). Both for partial and limit digestion, two spots were obtained on chromatograms and four spots, on electrophoretograms. The spots were eluted and analysed by high-voltage paper electrophoresis at pH 5.0 and 6.5 .They were identified as nucleosides 3′-phosphates, and no traces of the 5′- and 2′:3′-cyclic isomers were present. It should be noted that by electrophoresis in the buffer system used (acetic acid - pyridine - water at pH 3.5) it was possible to separate nucleoside 3′-phosphate from nucleoside 5′-phosphate, except uridine 3′- and 5′-phosphates which under these conditions were not separated.

DISCUSSION

Although after the chromatography on CM-Sephadex two fractions α' and α'' were obtained containing, respectively, 18 and 26% of the enzymic activity of the starting extract, only the properties of fraction α'' were studied. However, initial experiments with fraction α' showed that the enzymic properties of both fractions were identical. The enzyme is stable only between pH 5 and 7.5, and it is thermolabile. The attempts to purify it by ethanol and acetone fractionation were unsuccessful. Concentration of the enzyme preparation by freeze-drying was accompanied by the loss of activity up to 40%. Also concentration by the tannin-caffeine method (Mejbaum-Katzenellenbogen, 1959a,b) or dialysis against saccharose were unsuccessful. The enzyme can be stored frozen at -15° for several months. Repeated freezing and thawing do not destroy the activity.

The molecular weight of fraction α'' estimated by gel filtration was about 104 000. The enzyme was free of phosphatase, nucleotidase, phosphodiesterase and DNase activities. When the activity of pig pituitary RNase is expressed in units used for pancreatic RNase (McDonald, 1955a), then 1 g. of pig pituitary contains 550 units and 1 g. of bovine pancreas 220 units.

The study on the mechanism of hydrolysis showed that, in contrast to the pancreatic RNase, the pituitary enzyme hydrolysed RNA without formation of cyclic nucleotides. The enzyme is an exonuclease which hydrolyses RNA directly to 3′-nucleotides and shows no activity toward cytidine 2′:3′-cyclic phosphate. The mode of action of pituitary RNase resembles that of nucleases obtained from calf spleen (Heppel, Markham & Hilmoe, 1953), *Lactobacillus acidophilus* (Fiers & Khorana, 1963) and *Bacillus subtilis* (Kerr, Pratt & Lehman, 1965).

The pig pituitary RNase, similarly as the pancreatic enzyme, does not require activators. Both enzymes are inactivated by Hg^{2+} and Cu^{2+} ions, and their activity is inhibited by high concentrations of NaCl. The temperature optimum for both enzymes is similar: 60° for the pancreatic enzyme and 50° for the pituitary RNase. The pancreatic RNase is more affected by metal ions, e.g. Ca^{2+}, Mn^{2+} and Mg^{2+}.

Besides the difference in the mode of action and in molecular weight of these two enzymes, there is also a very big difference in their stability. The pancreatic enzyme, in contrast to the pituitary RNase, is very stable over a broad range of pH values and temperature.

The authors wish to express their gratitude to Professor Dr. Tadeusz Baranowski for his interest and advice, Dr. A. Szewczuk for his helpful discussions, Dr. Elwira Lisowska of the Department of Immunochemistry and Dr. M. Wolny of the Department of Biochemistry of the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wrocław for gifts of horse mucoid and glyceraldehydephosphate dehydrogenase, respectively.

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3′-NUKLEOTYDOHYDROLAZA RYBONUKLEINIANU Z PRZYSADEK MÓZGOWYCH ŚWINI

Streszczenie

1. Kwaśną rybonukleazę z przysadek mózgowych świni oczyszczono 45-krotnie. Preparat nie wykazuje aktywności fosfatazowej, nukleotydazowej, DNazowej i fosfodiesterazowej.

2. Enzym nie wymaga dla swojej aktywności jedno- i dwuwartościowych jonów metali; jego optimum pH wynosi 6,5.

3. Enzym jest egzonukleazą hydrolizującą drożdżowy RNA do 3′-mononukleotydów. W produktach hydrolizy nie znaleziono ani 5′- ani cyklicznych nukleotydów. Enzym nie hydrolizuje również cyklicznego 2′:3′-fosforanu cytydyny.

4. Ciężar cząsteczkowy enzymu wynosi 104 000.

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SYNTHESIS OF ARGININE FROM CARBAMOYL ASPARTATE AND ORNITHINE IN PREPARATIONS FROM WHEAT SEEDLINGS

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1. Synthesis of arginine from L-carbamoyl aspartate and L-ornithine in preparations from wheat plants was studied. 2. It was found that this synthesis required neither ATP, nor Mg^{2+}. 3. It seems that in this new path argininosuccinate synthesis is catalysed by a specific enzyme system, since the purified preparation catalysing arginine synthesis from citrulline and aspartate did not catalyse this reaction. 4. Acetone powder lost its activity for the new path after 10 days of storage, but for the classical path of arginine synthesis this same acetone powder retained full activity for 6 - 8 weeks. 5. Some kinetic characteristics of the new path are given.

The classical path of arginine synthesis from citrulline and aspartate has been described long ago (cf. Ratner, 1954). However, all studies reported in the literature were dealing with enzyme present in animal organs and micro-organisms. This path was also shown to operate in higher plants, as demonstrated on extracts from young wheat plants (Fotyma, Kleczkowski & Reifer, 1961) and on extracts from pea seedlings after separation on Sephadex G-25 of a natural inhibitor of arginine synthesis, present in pea homogenates (Grabarek, Reifer & Kleczkowski, 1965).

Della Pietra, Rogliani & Procaccini (1957) working with rat-liver preparations, have proposed a new path of arginine synthesis from ornithine and carbamoyl aspartate. However, this work has not been confirmed in other laboratories and the probability of this suggestion has been questioned (Crokaert, 1963).

In this report we present some results which would indicate that in preparations from young wheat plants such arginine synthesis is indeed possible.

MATERIAL AND METHODS

Reagents. L-Ornithine-HCl (Reanal, Budapest, Hungary); L- and DL-carbamoyl aspartic acid (Calbiochem, Los Angeles, Calif., U.S.A.); DL-α- and DL-β-methyl-aspartate, argininosuccinate-Ba and ATP-Na (Sigma, St. Louis, Mo., U.S.A); citrulline and urease, 250 units/mg. (Merck, Darmstadt, Germany). All other reagents were of Polish origin distributed by Biuro Obrotu Odczynnikami Chemicznymi (Gliwice). Arginase was prepared from bovine liver according to Ratner (1955).

Enzyme extract preparation for the synthesis of arginine from ornithine and carbamoyl aspartate: 6 - 8-day-old wheat, barley or oats plants were homogenized with 0.8 M-glucose - 1 M-Na-K-phosphate buffer, pH 7.2. The homogenate was filtered through four layers of cheese cloth and centrifuged for 1 hr. at 30 000 *g*. The sediment containing the enzyme activity was suspended in the above buffer and centrifuged again for 15 min. at 10 000 *g*. After centrifugation the sediment was discarded and the supernatant used as enzyme source. All steps of the procedure were carried out in a cool room at 4°.

The incubation mixture contained: 0.25 ml. of enzyme preparation (about 1.4 mg. protein); 15 μmoles L-ornithine-HCl; 20 μmoles DL-carbamoyl aspartate (10 μmoles L-carbamoyl aspartate); 55 μmoles Na-K-phosphate buffer, pH 7.2, in 0.8 M-glucose, and water to total volume of 0.8 ml. After incubation for 40 min. at 30° the samples were inactivated with 0.5 ml. of 1 N-HCl, mixed well and neutralized with 0.5 ml. of 1 N-NaOH, again mixed well, then urease (20 units), and arginase (20 units) added and incubated a second time for 30 min. at 37°. From the amount of liberated ammonia, arginine synthesis was calculated. The controls were prepared exactly as above, but without the addition of carbamoyl aspartate.

Enzyme extract preparation for the synthesis of arginine from citrulline and aspartate: the extract from 6 - 8-day-old wheat seedlings in 0.1 M-tris-maleate-NaOH buffer, pH 7.0, was fractionated with ammonium sulphate and the fraction between 0.5 and 0.7 saturation collected, dissolved in 0.2 M-K-phosphate buffer, pH 7.4, and again fractionated. The fraction between 0.55 - 0.7 sat. was dissolved in K-phosphate buffer as above, freed from ammonium sulphate on a Sephadex G-25 (medium) column, divided into 2 - 5 ml. portions and stored at -18° as enzyme source.

The incubation mixture for the synthesis of arginine from citrulline and aspartate contained: 60 - 100 μg. of enzyme protein, 5 μmoles each of L-citrulline, L-aspartate and ATP; 10 μmoles of $MgSO_4$, 100 μmoles of K-phosphate buffer, pH 7.4, and water to 0.8 ml. Time of incubation 40 min. at 37°. All further steps were as described above for the synthesis of arginine from ornithine and carbamoyl aspartate. The controls were prepared without citrulline and aspartate.

Argininosuccinate lyase activity was determined according to Ratner (1955).

Ammonia was determined using Conway vessels technique (Conway, 1947). Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951).

Enzyme activity was expressed in μmoles of arginine synthesized per mg. of protein per 40 min. at 30° or 37°.

The reaction product was identified as follows: the fourfold amount of enzyme preparation and substrates were incubated as above at 37°. The control sample was run parallel without carbamoyl aspartate. After 40 min., 0.5 ml. of 72% $HClO_4$ was added to each sample. The samples were cooled and neutralized with 4 N-KOH. After centrifugation supernatants were evaporated to dryness in vacuum. The residues were dissolved each in 1 ml. of water and chromatographed on Amberlite IR-120 (H^+ form) column (20×1.4 cm.). After washing the column with 300 ml. of water, elution was started with 150 ml. of 2 N-NH_4OH. The collected eluate

was evaporated on a boiling water bath to 2 ml. and chromatographed with appropriate standards (arginine, ornithine, carbamoyl aspartate, citrulline and argininosuccinate) on Whatman no. 1 paper. Three parallel chromatograms were run in phenol saturated with water solvent for 20 hr. and after drying sprayed with ninhydrin, isatin, and Sakaguchi reagent, respectively.

RESULTS

The results shown in Fig. 1 confirm the enzymic character of the reaction studied. Arginine synthesis from ornithine and carbamoyl aspartate was also confirmed by paper chromatography and subsequent spraying with amino acid reagents or specific reagent for the guanidine group. No arginine was found in the control samples. The synthesis was studied in 6 - 10-day-old plants of wheat, barley and

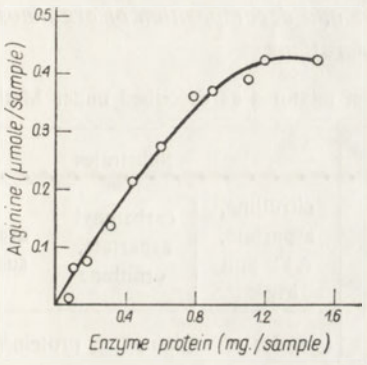


Fig. 1

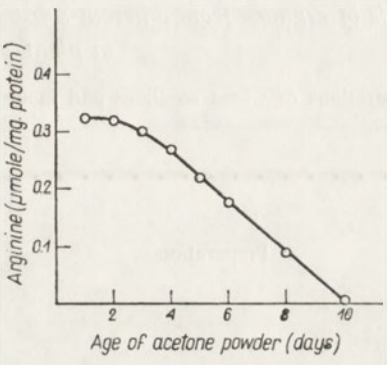


Fig. 2

Fig. 1. Effect of enzyme protein concentration on arginine synthesis. The incubation mixture was as described under Methods.

Fig. 2. Effect of storage of acetone powder from wheat seedlings on arginine-synthesizing activity. The incubation mixture was as described under Methods.

oats. The highest activity was found in wheat plants (Table 1). A dependence was observed between the age of wheat plants and arginine-synthesizing activity. Wheat plants were studied in the age from 2 to 25 days and the activity oscillated between 0.08 - 0.3 µmoles per mg. of protein. The highest activity was found in 6 - 8-day-old plants.

Acetone powder prepared from 6 - 8-day-old wheat seedlings lost its activity up to 50% after 6 - 7 days of storage at -18°, and after 10 days it was entirely inactive (Fig. 2). It may be of interest that the same acetone powder retained its full activity for the synthesis of arginine from citrulline and L-aspartate even when kept at 4° for 7 - 8 weeks.

The synthesis of arginine from carbamoyl aspartate and ornithine requires neither addition of ATP or Mg^{2+}, nor both of them together. The concentrations studied were: for Mg^{2+} 10 - 20 mM and for ATP 5 - 10 mM. The purified wheat

Table 1

Synthesis of arginine from carbamoyl aspartate and ornithine in plant seedlings homogenates

Incubation mixture as described under Methods.

Plant	Arginine synthesis (μmoles/mg. protein)
Wheat	0.235
Barley	0.092
Oats	0.047

Table 2

Synthesis of arginine from different substrates and decomposition of argininosuccinate in plant preparations

Preparations of wheat seedlings and incubation mixtures as described under Methods.

Preparation	Substrates		
	citrulline, aspartate, ATP and Mg^{2+}	carbamoyl aspartate, ornithine	arginino-succinate
	(μmoles of arginine/mg. protein/40 min.)		
Homogenate from 6-day-old wheat seedlings	0.82	0.24	—
Homogenate from 15-day-old wheat seedlings	0.36	0.09	—
Purified preparation for citrulline - aspartate path	10.11	0	5.95
Supernatant II for carbamoyl aspartate - ornithine path	0	0.51	3.84
Homogenate from 10-day-old etiolated wheat seedlings	0.50	0	—

seedlings preparation for arginine synthesis from citrulline and L-aspartate was not able to catalyse the arginine synthesis from ornithine and carbamoyl aspartate, whereas the preparation for the synthesis from ornithine and carbamoyl aspartate did not synthesize arginine from citrulline and L-aspartate. Both above preparations were active in cleavage of argininosuccinate to arginine and fumarate (Table 2). It seems interesting that preparations from etiolated pea seedlings have shown arginine synthesizing activity from citrulline and aspartate and were inactive with ornithine and carbamoyl aspartate as substrates (Table 2).

We have tried to repeat the work of Della Pietra *et al.* (1957) with rat-liver extracts under conditions described by those authors (Della Pietra, Rogliani, Rogliani

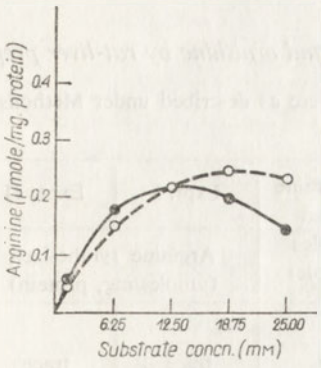


Fig. 3

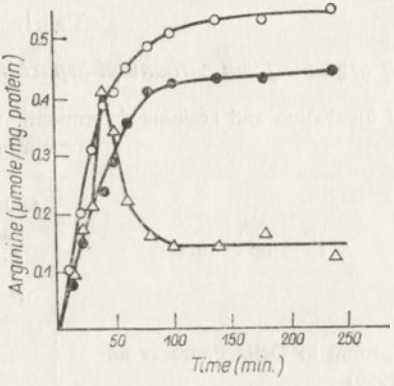


Fig. 4

Fig. 3. Effect of substrates concentration on arginine synthesis. The incubation mixture was as described under Methods. At indicated concentrations of ornithine 12.5 mM-carbamoyl aspartate and at indicated concentrations of L-carbamoyl aspartate 18.75 mM-ornithine were used. (●), Carbamoyl aspartate; (○), ornithine.

Fig. 4. Effect of temperature and incubation time on arginine synthesis. The incubation mixture was as described under Methods. Temperature: (●), 25°; (○), 30°; (△), 37°.

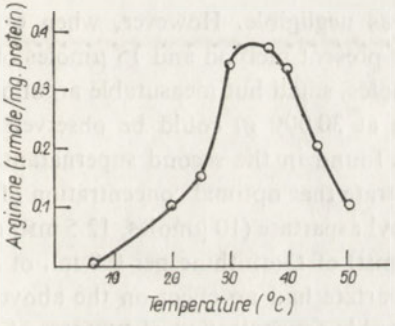


Fig. 5

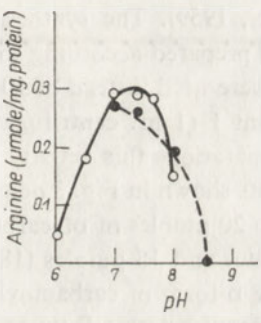


Fig. 6

Fig. 5. Effect of temperature on arginine synthesis. The incubation mixture was as described under Methods.

Fig. 6. Effect of pH on arginine synthesis. The incubation procedure and buffer concentration were as described under Methods. (○), Phosphate buffer; (●), tris-HCl buffer.

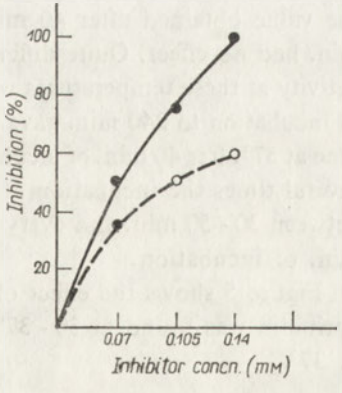


Fig. 7. Inhibitory effect of (●), DL-α- and (○), DL-β-methyl-aspartate on arginine-synthesizing activity. The incubation mixture was as described under Methods.

Table 3

Synthesis of arginine from carbamoyl aspartate and ornithine by rat-liver preparations

Procedure of incubation and arginine determination were as described under Methods for plant preparations

Enzyme extract	L-Ornithine added (μmoles /sample)	Expt. I	Expt. II
		Arginine synthesized (μmoles/mg. protein)	
According to Della Pietra *et al.* (1959)	10	trace	trace
	15	0.003	0.005
According to the presented method			
Supernatant I, 30 000 *g*	10	trace	trace
	15	0.053	0.055
Supernatant II, 10 000 *g*	10	0	0
	15	0	0

& Andreucci, 1959). The synthesis was negligible. However, when extract from rat liver was prepared according to the present method and 15 μmoles of ornithine per sample were used instead of 10 μmoles, small but measurable arginine synthesis in supernatant I (1 hr. centrifugation at 30 000 *g*) could be observed (Table 3). In plant preparations this activity was found in the second supernatant.

The results shown in Fig. 3 demonstrate that optimal concentration of substrates amounted to 20 μmoles of DL-carbamoyl aspartate (10 μmoles, 12.5 mM, of L-carbamoyl aspartate) and 15 μmoles (18.75 mM) of L-ornithine per 0.8 ml. of incubation mixture. The D-form of carbamoyl aspartate had no effect on the above described activity, as samples with L-form and double concentration of DL-form of carbamoyl aspartate gave the same results. Therefore double concentration of DL-form was used.

Interesting results were obtained by studying the effect of incubation time on arginine-synthesizing activity at 25, 30 and 37° (Fig. 4). At 37° after 40 min. of incubation a sharp decrease was found in arginine content up to 100 min. (1/3 of the value obtained after 40 min.). Further prolongation of incubation up to 240 min. had no effect. Quite different results were obtained at 25 and 30°. Maximum activity at these temperatures was obtained after 100 min. and further prolongation of incubation to 240 min. gave no change in arginine content. The decrease in arginine at 37° after 40 min. of incubation remains entirely unexplained. We have repeated several times the incubation at 37° even in the narrower range of incubation time between 30 - 50 min. and every time the peak of activity was found between 40 - 45 min. of incubation.

Figure 5 shows the effect of temperature on arginine-synthesizing activity. The optimum was found at 30 - 37°. At 50° the activity was only 1/4 of that obtained at 37°.

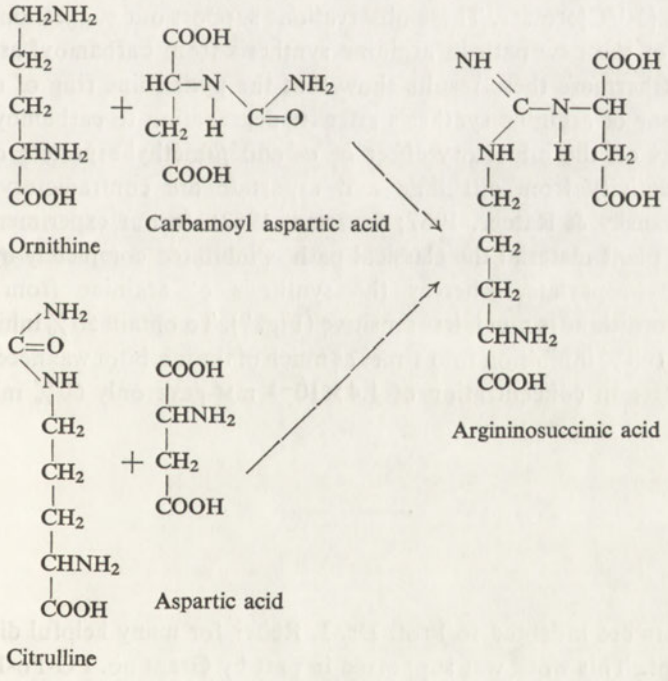
Optimum pH was found to be between 7.0 and 7.6 (Fig. 6) and phosphate buffer appeared to be better than tris-HCl.

The inhibition of arginine synthesis from carbamoyl aspartate and ornithine by DL-α- and DL-β-methyl-aspartate was also studied (Fig. 7). DL-α-Methyl-aspartate was a stronger inhibitor than the β-form; 50% of inhibition was achieved with 7×10^{-2} mM-DL-α-methyl-aspartate, but 1.5×10^{-1} mM-DL-β-methyl-aspartate was necessary to achieve the same range of inhibition. 1.4×10^{-1} mM-DL-α-methyl aspartate inhibited arginine synthesis in 100% but the β-form at this concentration only in 60%.

DISCUSSION

In the above described studies we have presented some results suggesting the occurrence in higher plants of a new path of arginine-synthesizing activity from ornithine and carbamoyl aspartate. According to our findings this new path appears to be catalysed by an enzyme system independent from the classical path in which citrulline and aspartate are required as substrates. These observations refer only to the first step in arginine synthesis related to the formation of argininosuccinate (Table 2).

It is clear from the chemical point of view, that the condensation product of ornithine and carbamoyl aspartate as well as citrulline and aspartate must be argininosuccinate, as presented in Scheme 1.



Scheme 1.

It seems that the synthesis of argininosuccinate in higher plants is catalysed by two independent enzyme systems, whereas the cleavage of argininosuccinate is catalysed by one enzyme.

The synthesis of arginine from carbamoyl aspartate and ornithine seems to be of interest as it appears not to require ATP and Mg^{2+}, as is the case in the classical path of arginine synthesis from citrulline and aspartate.

The part played by the supplementary path described above is not at all negligible. In 6-day-old wheat seedlings homogenates (Table 2) the citrulline - aspartate path was responsible for 0.82 μmole of synthesized arginine per mg. protein and the carbamoyl aspartate - ornithine path for 0.24 μmole per mg. protein. It is difficult to compare the results obtained with supernatant II for the new path (0.51 μmole) with those for the partially purified preparation for the classical path (10.1 μmoles) because the latter preparation was purified about tenfold whereas the former only twofold.

The activity for the new path of arginine synthesis was 5 to 10 times higher in plants than in rat-liver preparations when calculated per mg. protein.

The new path may be of certain advantage in the cell as it does not require additional chemical energy stored in ATP. Furthermore, since carbamoyl aspartate is a degradation product of dihydro-orotate appearing also in plants (Mazuś & Buchowicz, 1966) it may be directly utilized in arginine synthesis.

Wasternack & Reinbothe (1967) studying the metabolism of ureido-[^{14}C]carbamoyl aspartate and [6-^{14}C]- and [2-^{14}C]orotate in caps of *Agaricus bisporus* have found in urea 12% of ^{14}C from carbamoyl aspartate, 7% from [2-^{14}C]orotate and only 2% from [6-^{14}C]orotate. These observations support our suggestion concerning the existence of the new path in arginine synthesis from carbamoyl aspartate and ornithine. Furthermore these results show that the pyrimidine ring of orotate may be the precursor of arginine synthesis after its degradation to carbamoyl aspartate.

The reports on the inhibitory effect of α- and β-methyl-aspartate on arginine-synthesizing activity from citrulline and aspartate are contradictory (Crokaert, 1963; Rochovansky & Ratner, 1967; Severina, 1962). In our experiments we have found that in plant material the classical path is inhibited completely by 3.3×10^{-2} mM-DL-α-methyl-aspartate, whereas the synthesis of arginine from carbamoyl aspartate and ornithine is much less sensitive (Fig. 7). To obtain 50% inhibition twice as much, and 100% inhibition four times as much of the inhibitor was necessary. DL-β-Methyl-aspartate in concentration of 1.4×10^{-1} mM gave only 60% inhibition.

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SYNTEZA ARGININY Z KARBAMOILOASPARAGINIANU I ORNITYNY W PREPARATACH Z KIEŁKÓW PSZENICY

Streszczenie

1. Badano syntezę argininy z karbamoiloasparaginianu i ornityny w preparatach z kiełków pszenicy.

2. Stwierdzono, że synteza ta nie wymaga obecności ATP i Mg^{2+}.

3. Przypuszcza się, że ta nowa droga syntezy argininy jest katalizowana przez specyficzny układ enzymatyczny, ponieważ oczyszczone z kiełków pszenicy preparaty syntetyzujące argininę według klasycznej drogi (z cytruliny i asparaginianu), nie katalizują nowej drogi syntezy argininy — i odwrotnie, aktywne preparaty syntetyzujące argininę z ornityny i karbamoiloasparaginianu nie katalizują klasycznej drogi syntezy argininy.

4. Wykazano, że tylko synteza argininobursztynianu jest katalizowana w obu drogach przez różne układy enzymatyczne. Rozpad natomiast argininobursztynianu katalizują obydwa preparaty w jednakowym stopniu.

5. Opracowano optymalne warunki dla syntezy argininy z ornityny i karbamoiloasparaginianu.

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METABOLISM OF NUCLEOSIDE DIPHOSPHATE SUGARS

SEPARATION OF NUCLEOSIDE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASES OF *S. TYPHIMURIUM* BY GEL FILTRATION ON SEPHADEX G-200

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1. The formation of nucleoside diphosphate sugars from glucose-1-[^{32}P]phosphate and various nucleoside-5′-triphosphates was studied in extracts of *S. typhimurium* cells. 2. On fractionation of the extracts on Sephadex G-200 the pyrophosphorylases studied were partially separated and their molecular weights were calculated to be: for the enzymes specific for TPDglucose ~50 000, specific for CDPglucose and UDPglucose ~100 000, and specific for ADPglucose and GDPglucose above 200 000.

The occurrence in *Enterobacteriaceae* of a variety of nucleoside diphosphate sugars differing both in the sugar and nucleoside portions is related to the biosynthesis of specific polysaccharides of the O-antigen. The genus *Salmonella* has been studied most extensively in this respect (Nikaido, Naido & Mäkelä, 1966).

The aim of the present paper was to demonstrate that the very similar reactions of glucose-1-phosphate with various nucleoside-5′-triphosphates resulting in various nucleoside diphosphate sugars are catalysed by separate proteins. The choice of *Salmonella typhimurium* stemmed from the fact that in this species the genetic map, especially of the pyrophosphorylases studied, is well known and also from the availability of a variety of mutants for further studies.

EXPERIMENTAL

Material. S. typhimurium Lt2 was grown overnight at 37° in a medium described by Vogel & Bonner (1956), supplemented with rare elements (Ames & Carry, 1960). The cells were harvested by centrifuging at 7000 *g* for 15 min., washed three times with physiological saline, suspended in 0.2 M-phosphate buffer, pH 7.4, and disrupted by sonication (4 min., 24 kHz). The sonicated suspension was centrifuged at 10 000 *g* for 20 min. The clear supernatant containing about 15 mg. protein per ml. was used for fractionation studies without further treatment.

Chemicals. ATP, GTP and TMP (sodium salts) were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). CTP, potassium salt, and UTP, sodium salt, were from CalBiochem. (Los Angeles, Calif., U.S.A.). Glucose-1-phosphoric acid dipotassium salt was from Reanal Finomvegyszergyar (Budapest, Hungary). Sephadex G-200 (particle size 40 - 120 μ) was Pharmacia Ltd. (Uppsala, Sweden) product. Charcoal (Zakł. Elektr. Węgl., Racibórz, Poland) was prepared according to Threlfall (1957). Protein standards for molecular weight estimations were: ribonuclease (mol. wt. 13 700), L. Light & Co. Ltd. (Colnbrook, England); human albumin (mol. wt. 69 000) and human γ-globulin (mol. wt. 150 000), products of Warsaw Serum and Vaccine Plant (Poland). [^{32}P]Orthophosphate was a product of Institute of Nuclear Research (Warszawa, Poland).

Thymidine-5′-triphosphate was synthetized by the method of Moffat (1964) from TMP-morpholidate and pyrophosphate. TMP-morpholidate was prepared from TMP according to the procedure of Moffat & Khorana (1961). The isolation of TTP from the reaction mixture was performed by column chromatography on Dowex-1-chloride (Smith & Khorana, 1958), and included the concentrating of the pooled UV-absorbing peak on a small column of the same resin and precipitation of barium salt with ethanol (Canellakis, Gottesman, Kamen & Irvin, 1962). The product exhibited the UV to phosphorus ratio of 1:3.5 (theor. 1:3). The solution of sodium salt of TTP was obtained by precipitation of barium with sodium sulphate.

Glucose-1-[^{32}P]phosphate was prepared by enzymic phosphorolysis of starch using potato juice as the source of enzyme and [^{32}P]phosphate buffer (McCready, Hassid & Lardy, 1955). The isolation and purification of the product involved the removal of unreacted phosphate with magnesium acetate and ammonia, column chromatography on Dowex-50 (H^+) and Amberlite-IR 4B (OH^-), elution from the latter with 5% KOH and crystallization from the alkaline eluate by adding 3 vol. of methanol and a few drops of ether. The yield was 10% of the amount of [^{32}P]phosphate used. On paper chromatography (Sawicka, 1967) the product exhibited a single, radioactive spot identical with that of standard glucose-1-phosphate. The specific activity of glucose-1-[^{32}P]phosphate used for experiments varied from 2×10^5 to 2×10^6 counts/min./μmole.

Analytical. Phosphorus assays were carried out by the method of Fiske & Subbarow (1925) for amounts of 10 - 50 μg. P, and by the method of Ernster, Zetterström & Lindberg (1950) for those below 10 μg. P. The determination of ^{32}P was performed using a G.M. mica-end window counter and conventional ancillary equipment. Protein was estimated spectrophotometrically by measuring the extinction at 280 mμ, and by the method of Lowry, Rosebrough, Farr & Randall (1951).

Gel filtration. The centrifuged sonicated extract, 1 ml., was applied to a 1.4×60 cm. column of Sephadex G-200 and eluted with 0.145 M-NaCl - 0.005 M-tris-HCl buffer, pH 7.5, at a rate of about 12 ml. per minute. Fractions of 1.7 ml. were collected with the aid of a fraction collector.

Enzyme assay. Reaction mixture contained the following components in a total volume of 0.6 ml.: glucose-1-[^{32}P]phosphate (10^4 - 10^5 counts/min.), 0.05 μmole;

nucleoside-5′-triphosphate (ATP, CTP, GTP, UTP or TTP), 0.1 μmole; tris-HCl buffer, pH 7.5, 20 μmoles; $MgCl_2$ and enzyme preparation: 0.02 ml. of crude supernatant or 0.2 ml. of the eluate from Sephadex column. The tubes were incubated for 30 min. at 37°. The reaction was stopped by adding 0.6 ml. of 10% TCA and treated with 1 ml. of an aqueous suspension of charcoal (50 mg./ml.). The amount of labelled nucleoside diphosphate sugar formed was estimated as described elsewhere (Sawicka & Chojnacki, 1968).

RESULTS

Figure 1 represents a fractionation of the extract of *S. typhimurium* on a column of Sephadex G-200. A clear separation of three groups (*1*, *2*, *3*) of pyrophosphorylases was obtained. ADPglucose pyrophosphorylase and GDPglucose pyrophosphorylase emerged first from the column as closely coinciding peaks (*1*). They came off after the bulk of high molecular weight proteins. They were followed by CDPglucose

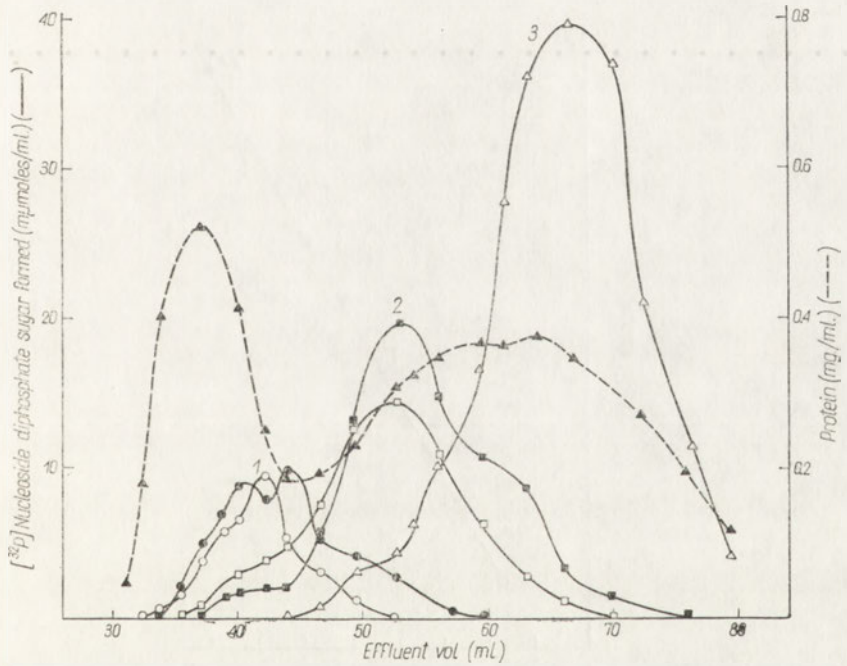

Fig. 1. Fractionation of nucleoside diphosphate glucose pyrophosphorylases of *S. typhimurium* by gel filtration on Sephadex G-200. 10 000 *g* supernatant of the sonicated suspension of bacterial cells (1 ml.) was applied to a 1.4×60 cm. column of Sephadex G-200 and eluted with 0.145 M-NaCl - 0.005 M-tris-HCl, pH 7.5. Fractions of 1.7 ml. were collected.

Pyrophosphorylases of: (○), ADPglucose; (□), CDPglucose; (●), GDP glucose; (■), UDPglucose; (△), TDPglucose. All activities are expressed in mμmoles of synthetized nucleoside diphosphate sugar per 1 ml. of eluate. (▲), Amount of protein (mg./ml.).

pyrophosphorylase and UDPglucose pyrophosphorylase eluted together (*2*) and by a large peak of TDPglucose pyrophosphorylase (*3*). The character of the peaks of the first two enzymes indicates that ADPglucose pyrophosphorylase and GDPglucose pyrophosphorylase are separate enzymes. The latter forms two discrete peaks which are slightly shifted on- and backwards in respect to ADPglucose pyrophosphorylase. The peaks of CDPglucose- and UDPglucose pyrophosphorylase do not coincide exactly with each other, the latter being slightly shifted onwards and showing irregular character in the right part of the peak. This might indicate the existence of more than one UDPglucose pyrophosphorylase. The peak of TDPglucose pyrophosphorylase is clearly separated from the others.

For molecular weight determination (Fig. 2), the Sephadex G-200 columns were standardized with pure proteins of known molecular weight. The values calculated for the enzymes studied are: ADPglucose pyrophosphorylase and GDPglucose pyrophosphorylase, above 200 000; CDPglucose pyrophosphorylase and UDPglucose pyrophosphorylase, about 100 000, and TDPglucose pyrophosphorylase, about 50 000.

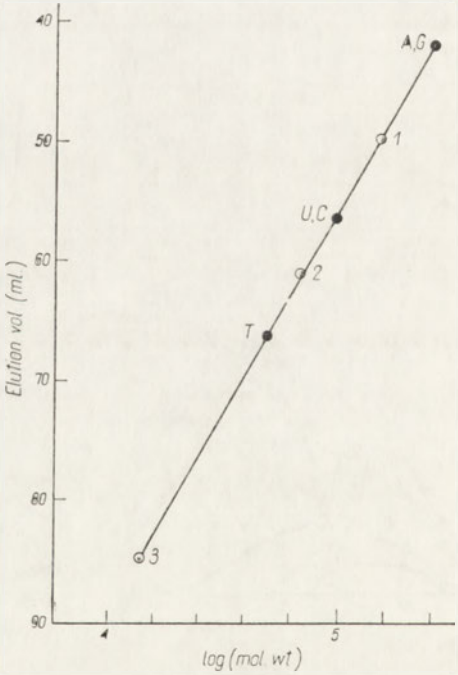

Fig 2. Determination of molecular weights of nucleoside diphosphate sugar pyrophosphorylases of *S. typhimurium* by Sephadex G-200 gel filtration. In the eluate from a 1.4×60 cm. column, the elution volumes of enzymes studied were compared with those of proteins of known molecular weight as described by Andrews (1964). Standard proteins: *1*, human γ-globulin; *2*, human albumin; and *3*, RNase; *A*, *C*, *G*, *U* and *T*, adenosine-, cytidine-, guanosine-, uridine- and thymidine diphosphate glucose pyrophosphorylases, respectively.

DISCUSSION

Preliminary experiments on the occurrence of nucleoside diphosphate sugar pyrophosphorylases in various biological materials (in preparation) have shown that the extracts of *S. typhimurium* contain one of the widest enzyme spectra in respect to the number of potent nucleoside-5′-triphosphates that activate glucose-1-phosphate. It was similar to that obtained in *E. coli* B and *E. coli* K_{12}.

The elution pattern of TDPglucose pyrophosphorylase of *S. typhimurium* from Sephadex G-200 obtained in this paper strikingly resembles that reported by Bernstein & Robbins (1965) for *E. coli* B enzyme. The above authors separated it from UDPglucose pyrophosphorylase, but did not calculate the molecular weight, and did not assay the enzymes synthetizing ADPglucose, CDPglucose and GDPglucose. In our studies the molecular weight of TDPglucose pyrophosphorylase was about 50 000 and the enzyme was also easily separable from the other four pyrophosphorylases studied, by gel filtration on Sephadex G-100. The two pyrophosphorylases of CDPglucose and UDPglucose have similar molecular weights, about 100 000. Molecular weights of ADPglucose pyrophosphorylase and GDPglucose pyrophosphorylase could not be estimated exactly because of the limited capacity of the gel. They are probably in the range of 200 000 - 300 000.

The distribution of individual pyrophosphorylases (Fig. 2) shows that the lowest molecular weight concerns TDPglucose synthetase only. The medium molecular weight is represented by pyrophosphorylases that use pyrimidine (except thymine) nucleotides (C and U) and the highest molecular weight is represented by pyrophosphorylases that use purine nucleotides (A and G).

The observed irregularity of elution profile of GDPglucose pyrophosphorylase and UDPglucose pyrophosphorylase that suggests the existence of more than one enzymic activity of a given type, is in accord with the results of Nikaido, Nakane & Levinthal (1967) who found separate UDPglucose pyrophosphorylases in *S. typhimurium* by chromatography on DEAE-cellulose.

Our thanks are due to Doc. Dr. T. Kłopotowski for providing the strain of *S. typhimurium* and for helpful discussion.

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METABOLIZM NUKLEOZYDODWUFOSFOCUKRÓW

ROZDZIELANIE PRZY POMOCY SEFADEKSU G-200 PIROFOSFORYLAZ NUKLEOZYDODWUFOSFOCUKRÓW *SALMONELLA TYPHIMURIUM*

Streszczenie

1. Aktywność pirofosforylaz nukleozydodwufosfocukrów w ekstraktach *S. typhimurium* badano mierząc inkorporację glukozo-1-[^{32}P]fosforanu do materiału nukleotydowego adsorbującego się na węglu aktywnym w obecności ATP, CTP, GTP, UTP i TTP.

2. Za pomocą filtracji na żelu Sefadeks G-200 oddzielono pirofosforylazę tymidynodwufosfoglukozy od pirofosforylaz cytydynodwufosfoglukozy i urydynodwufosfoglukozy, eluowanych z kolumny we wspólnym szczycie, oraz pirofosforylaz adenozynodwufosfoglukozy i guanozynodwufosfoglukozy wymywających się razem. Określono masy cząsteczkowe badanych enzymów.

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THE EFFECT OF TRITON X-100 ON RESPIRATORY CHAIN OF RAT-LIVER MITOCHONDRIA

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1. Triton at increasing concentration decreases gradually the respiratory control of tightly coupled mitochondria. The decrease is accompanied by activation of the Mg^{2+}-dependent ATPase and inhibition of the DNP-stimulated one. 2. Succinate oxidation with oxygen or cytochrome *c* as acceptor is inhibited by Triton concentrations almost ten times as high as those which inhibit choline oxidation. 3. When phenazine methosulphate is used as acceptor, Triton has no effect on the oxidation either with succinate or choline as substrates.

In our previous studies (Drabikowska & Szarkowska, 1965; Drabikowska, 1967) on the ability of different flavoenzymes containing non-haeme iron to reduce exogenous ubiquinone, the assay medium was always supplemented with the non-ionic detergent Triton X-100 assuming that Triton causes dispersion of water-insoluble ubiquinone (UQ_6) and enhances the accessibility of the acceptor to the enzyme. It has been repeatedly observed that reduction of ubiquinone increases with increasing Triton concentration.

Since the mechanism of action of the surface-active agents on cellular and subcellular structures is still obscure it seemed of interest to investigate the effect of Triton on the entire respiratory chain.

EXPERIMENTAL

Rats of Wistar strain weighing 200 - 220 g. were used for experiments. Rat-liver mitochondria were prepared by the method of Schneider & Hogeboom (1950) except that 0.25 M-sucrose - 10 mM-triethanolamine hydrochloride (TRA-HCl) - 0.5 mM-EDTA was used as medium (Bodie & Klingenberg, 1965). The mitochondrial pellet was kept at 0° in an ice bath before use.

The oxygen uptake and the respiratory control were assayed with Clark oxygen electrode. Consumption of oxygen with phenazine methosulphate as acceptor was determined by standard Warburg method under conditions described by Singer & Kearney (1957).

The reduction of cytochrome *c* was measured in the recording Eppendorf photo-

meter. The activity was calculated as described previously (Drabikowska & Szarkowska, 1965).

The ATPase activity was estimated as the amount of P_i released from ATP, P_i being determined by the method of Lowry & Lopez (1946).

The extent of lysis of mitochondria was measured by the decrease in extinction at 546 mμ.

Protein was determined by the biuret method as described by Szarkowska & Klingenberg (1963).

The composition of the various media used and the conditions of incubation are described in the legends of the tables and figures.

Triton X-100 solutions were always freshly prepared. From a stock solution of 20 mg. in 1 ml. of water appropriate dilutions were made.

All chemicals used were of reagent grade. ATP was a C.F. Boehringer & Soehne GmbH (Mannheim, Germany) product; Triton X-100 was kindly given by Rohm & Haas (Philadelphia, Pa., U.S.A.); phenazine methosulphate, choline and serum albumin were from Sigma Chem. Comp. (St. Louis, Mo., U.S.A.). Other reagents were of Polish origin.

RESULTS

ATPase activity. In tightly-coupled rat-liver mitochondria Mg^{2+}-dependent ATPase activity is latent and only a small amount of P_i is released from ATP. The addition of Triton to the mitochondrial suspension (Fig. 1) significantly increases P_i liberation with a maximum at 930 μM-Triton concentration (0.6 mg./ml.).

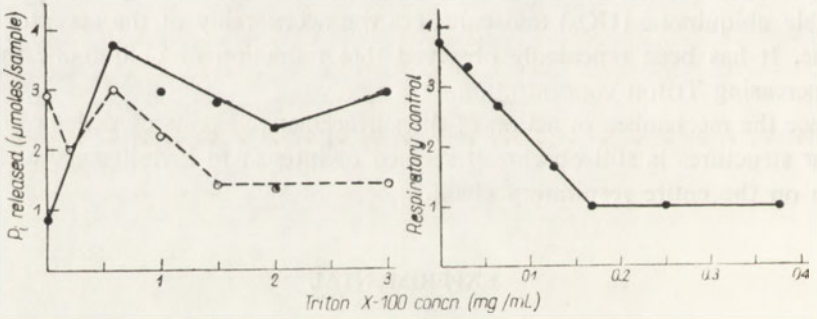

Fig. 1 Fig. 2

Fig. 1. The effect of Triton X-100 on the activity of: (●), Mg^{2+}-dependent ATPase, and (○), ATPase stimulated by DNP. Conditions: 70 mM-KCl, 40 mM-tris-HCl, pH 7.4; 12.5 mM-sucrose; 4 mM-ATP; 3 mM-$MgCl_2$ or 0.1 mM-DNP; and mitochondrial protein, 2.3 mg./ml. Final volume, 1 ml.; incubation 15 min. at room temperature.

Fig. 2. The effect of Triton concentration on respiratory control. Reaction mixture contained: 17 mM-TRA-HCl (triethanolamine hydrochloride), pH 7.2, 0.25 M-sucrose, 2 mM-inorganic phosphate, 2 mM-$MgCl_2$, and mitochondrial protein, 2 mg./ml. The consumption of oxygen was measured with 2 mM-succinate alone and after addition of 0.2 mM-ADP. Respiratory control is expressed as the ratio of the oxidation rate in the active state to the oxidation rate in the controlled state of mitochondria.

In the presence of dinitrophenol (DNP) the character of the curve of P_i liberation is quite different. Low concentration of Triton causes a 30% decrease in the amount of P_i released but at the concentration of 0.6 mg./ml. the amount of P_i found is equal to that observed in the absence of this detergent. Further increase in Triton concentration results first in a renewed decrease of P_i liberation and over 1.5 mg./ml. the P_i liberation remains at a constant level.

Respiratory control and the rate of oxidation. The addition of Triton X-100 in amount of 0.031 mg./ml. (48 μM) to the tightly coupled mitochondria decreases their respiratory control with succinate as substrate. The respiratory control decreases linearly with the increase in Triton concentration (Fig. 2) and is abolished at a concentration of 0.168 mg./ml.

The results presented in Table 1 show the effect of Triton on oxidation rates of various substrates in different metabolic states of mitochondria. The addition of Triton up to 0.125 mg./ml. (190 μM) is without effect on oxidation rate of succinate by mitochondria in active or uncoupled state. In the controlled state the rate of oxidation of succinate is increased by a factor of 2 in relation to the original activity. The addition of albumin to the mitochondria supplemented with Triton (0.125 mg./ml.) restores the respiratory control to the initial value. In the presence of higher concentration of Triton the difference in the oxidation rate between the three metabolic states investigated is abolished. However, a decrease in respiration rate in active and uncoupled states of mitochondria as compared with the initial values is observed. Under these conditions the addition of albumin has no effect.

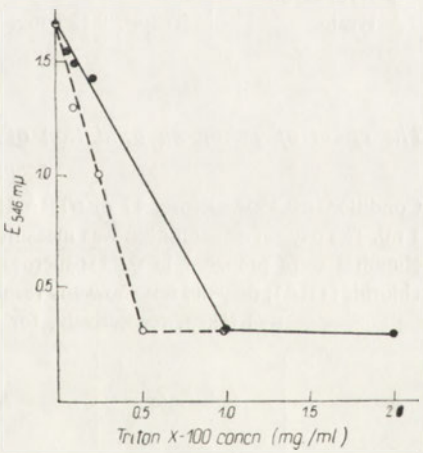

Fig. 3. Effect of Triton X-100 on lysis of mitochondria. Conditions as described in the legend of Table 1. The measurements of lysis were carried out as described in the Experimental section. (●), Mitochondria with respiratory control; (○), mitochondria without respiratory control.

Oxidation of glutamate and β-hydroxybutyrate in mitochondria uncoupled by DNP is inhibited by much lower concentration of Triton compared with oxidation of succinate. Oxidation of glutamate is inhibited by a Triton concentration of 0.031 mg./ml. in about 50% and of β-hydroxybutyrate in about 25%.

To follow the effect of Triton on choline oxidation, mitochondria without respiratory control were used. In fresh, coupled rat-liver mitochondria it was impos-

Table 1

Oxidation rates of various substrates by mitochondria in different metabolic states, in the presence of Triton X-100

The standard medium contained in 1 ml.: 0.25 M-sucrose, 17 mM-triethanolamine hydrochloride (TRA), pH 7.2, 2 mM-phosphate, 6 mM-substrate, 2 - 3.5 mg. of mitochondrial protein, and where indicated 0.3 mM-ADP and 0.05 mM-dinitrophenol. The oxygen consumption was measured with an oxygen electrode at room temperature.

Substrate	Addition	Oxygen consumption (mμmoles O_2/min./mg. protein)		
			with ADP	with DNP
Succinate	None	13	50	72
Succinate	Albumin, 7.5 mg.	9	42	44
Succinate	Triton, 0.031 mg.	16	52	80
Succinate	Triton, 0.125 mg.	27	45	75
Succinate	Triton, 0.125 mg.; albumin, 7.5 mg.	11	48	48
Succinate	Triton, 0.5 mg.	36	—	34
Succinate	Triton, 0.5 mg.; albumin, 7.5 mg.	34	34	34
Glutamate	None	4.5	—	24
Glutamate	Triton, 0.031 mg.	—	—	11
β-Hydroxybutyrate	None	6.4	—	38
β-Hydroxybutyrate	Triton, 0.031 mg.	—	—	29
β-Hydroxybutyrate	Triton, 0.125 mg.	—	—	5

Table 2

The effect of Triton on oxidation of succinate and choline by mitochondria without respiratory control

Conditions: 0.25 M-sucrose, 17 mM-TRA-HCl, pH 7.6, and 6 mM-substrate in a total volume of 1 ml. The oxygen consumption was measured with an oxygen electrode at room temperature. Mitochondria were prepared in 0.25 M-sucrose medium without EDTA and triethanolamine hydrochloride (TRA); they did not show any respiratory control; 1.83 or 5.2 mg. of mitochondrial protein was taken, respectively, for experiments with succinate and choline.

Substrate	Addition	Oxygen consumption (mμmoles O_2/min./mg. protein)
Succinate	None	41
Succinate	Triton, 0.125 mg.	30
Succinate	Triton, 0.5 mg.	20
Succinate	Triton, 1 mg.	10
Succinate	Triton, 3 mg.	4
Choline	None	10.8
Choline	Triton, 0.0156 mg.	6.7
Choline	Triton, 0.0312 mg.	3.3

sible to demonstrate choline oxidation because of the impermeability of mitochondria to this substrate. As shown in Table 2, the oxidation of choline is inhibited by very low concentration of Triton.

Triton additions bring about a decrease in extinction of the mitochondrial suspension which is much faster in mitochondria without respiratory control (Fig. 3). The swelling and subsequent lysis of mitochondria in the presence of Triton take place much faster than the complete inhibition of succinate oxidation. However, the oxidation of choline is markedly inhibited even before the changes in extinction become noticeable.

Reduction of cytochrome c. The reduction of exogenous cytochrome *c* in the presence of succinate and choline as substrates is shown in Table 3. Inhibition of cytochrome *c* reduction with succinate as substrate occurs at relatively high Triton concentration. On the contrary, reduction of cytochrome *c* by choline is inhibited by Triton concentrations about ten times lower than those used with succinate.

The activity of succinate and choline dehydrogenases. The activity of both dehydrogenases in mitochondria assayed by phenazine methosulphate method is not inhibited by Triton even in concentration of 1 mg./ml. (Table 4). The slight inhibition occurring occasionally seems to be insignificant.

DISCUSSION

In the present work the effect of Triton X-100 on electron transport and ATPase activity in mitochondria has been investigated. In tightly coupled mitochondria Triton at a low concentration evokes a marked increase in the rate of electron transport from succinate to oxygen, accompanied by a loss of respiratory control. The uncoupling effect of Triton can be abolished by albumin, which restores the respiratory control.

Albumin is well known as a factor binding fatty acids and therefore restoring the respiratory control (Boyer, Ballon & Luck, 1947; Good, 1958). Its effect on Triton-treated mitochondria seems to be similar but the mechanism is not known. It is possible that albumin also binds Triton and therefore abolishes its effect.

The stimulation of controlled respiration of mitochondria is accompanied by a simultaneous activation of Mg^{2+}-dependent ATPase and inhibition of the DNP-stimulated ATPase. A somewhat similar effect was observed by Witter & Mink (1958) at very low concentration of other non-ionic detergents. The effect of Triton on activities of ATPases was investigated over a wider range of its concentration (Fig. 1). A similar effect of long-chain fatty acids was reported first by Bos & Emmelot (1962), then by Wojtczak & Załuska (1967) and Erecińska (1967). This would imply that Triton either might stimulate liberation from membraneous lipids of fatty acids which in turn would influence the activity of the ATPases, or that it acts in the same manner as these acids. It may be also envisaged that Triton is adsorbed to the mitochondrial membranes causing some alterations in their structure, leading to formation of "pores". The occurrence of "pores" in Triton-

Table 3

The effect of Triton on cytochrome c *reduction*

Conditions: 50 mM-phosphate buffer, pH 7.2, 41 μM-cytochrome *c*, 1 mM-KCN, 2 mM-substrate and mitochondrial suspension in a total volume of 1 ml. The reaction was carried out at room temperature. In the experiment with succinate 0.196 mg. of mitochondrial protein was used, and in the experiment with choline twice as much.

Substrate	Addition	Reduced cytochrome *c* (mμmoles/min./mg. protein)	Inhibition (%)
Succinate	None	166	—
Succinate	Triton, 0.1 mg.	94	44
Succinate	Triton, 0.2 mg.	50	70
Choline	None	64	—
Choline	Triton, 0.01 mg.	2	97
Choline	Triton, 0.02 mg.	0	100

Table 4

The effect of Triton on choline and succinate dehydrogenases in rat-liver mitochondria

Conditions: each Warburg flask contained 125 mM-sucrose, 10 mM-TRA-HCl, pH 7.2 for succinate as substrate and pH 7.6 for choline, 50 μM-dinitrophenol, 0.1 mM-KCN, 0.2 ml. of 1% phenazine methosulphate, mitochondrial protein, and 0.2 ml. of 10% KOH in the center well. Total volume 3 ml. The consumption of oxygen was measured at 37° for 10 min. The results are expressed as μl. of oxygen/mg. of protein/10 min.

Substrate	Addition	Experiment no.	
		1	2
Choline	None	9.8	8.4
Choline	Triton, 0.33 mg./ml.	9.3	—
Choline	Triton, 0.66 mg./ml.	8.7	—
Choline	Triton, 1 mg./ml.	—	8.2
Choline	Triton, 2 mg./ml.	8.5	7.0
Succinate	None	15.6	14
Succinate	Triton, 1 mg./ml.	16.0	13
Succinate	Triton, 2 mg./ml.	—	12

treated chloroplasts was postulated by Deamer & Crofts (1967). If this is the case, the displacement of membrane components may lead to unmasking of some "latent" mitochondrial enzymes. Since the uncoupling effect of Triton and the activation of Mg^{2+}-dependent ATPase are accompanied by a decrease in extinction of the mitochondrial suspension, this seems to indicate that indeed some structural changes are due to the action of Triton itself.

Another effect of Triton was also observed. Following the initial stimulation of respiration (measured with oxygen as terminal acceptor) Triton at higher concentra-

tion causes a progressive decrease of the oxidation and finally its complete inhibition. It seems likely that Triton at first only slightly damages the functional lipid-protein link of individual dehydrogenases with the respiratory chain and then at higher concentration breaks the electron flow.

It should be noted that Triton has an about tenfold stronger inhibitory effect on choline oxidation than on succinate oxidation either with oxygen or cytochrome *c* as acceptors. The action of Triton on choline oxidase is very pronounced even at so low concentration of Triton that it gives almost no change in extinction and probably causes but a slight damage to the mitochondrion.

Both dehydrogenases, as it has been shown by Packer, Estabrook, Singer & Kimura (1960), have a common chain beginning from cytochrome *c*, and probably share the same pool of ubiquinone (Szarkowska, 1964), therefore the differences in their behaviour may be due only to various binding mechanism of the respective dehydrogenases to the respiratory chain. This suggestion seems to be supported by the observation that with phenazine methosulphate as acceptor no differences appeared between the activities of the two dehydrogenases in the presence of Triton. It is also consistent with the conclusion of Rendina & Singer (1959) that there is a difference in binding of choline and succinate dehydrogenases to the respiratory chain. Whereas the former enzyme can be hardly released in soluble form by phospholipase A, the latter is readily extracted with alkaline buffers from solvent-treated mitochondria (Singer, Kearney & Bernath, 1956). The authors suggest that choline dehydrogenase is linked to the chain by phospholipid bridges whereas succinate dehydrogenase by another kind of lipids. In view of the presented results it could be supposed that these lipids would be less susceptible to the action of Triton than those which bind choline dehydrogenase.

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WPŁYW TRITONU X-100 NA ŁAŃCUCH ODDECHOWY MITOCHONDRIÓW WĄTROBY SZCZURA

Streszczenie

1. Mitochondria wątroby szczura tracą stopniowo kontrolę oddechową w miarę wzrostu stężenia Tritonu w mieszaninie reakcyjnej. Utracie kontroli towarzyszy aktywowanie ATPazy stymulowanej jonami Mg^{2+} i hamowanie ATPazy stymulowanej dwunitrofenolem. Wyższe stężenia Tritonu hamują transport elektronów z bursztynianu i choliny na tlen i na egzogenny cytochrom *c*.

2. Stężenie Tritonu potrzebne do zahamowania w tym samym stopniu utleniania bursztynianu co choliny jest dziesięć razy niższe w przypadku choliny jako substratu.

3. W obecności fenazyny jako akceptora Triton nie wykazuje hamującego wpływu na utlenianie żadnego z tych substratów.

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RECENZJE KSIĄŻEK

RESEARCH IN PROTOZOOLOGY (Tze-Tuan Chen, ed.) vol. 2. Pergamon Press, Oxford 1967; str. 398.

Tom drugi *Research in protozoology* jest dalszym ciągiem czterotomowego wydawnictwa obejmującego różne nowoczesne problemy współczesnej protistologii opracowane przez wielu autorów pod ogólną redakcją Tze-Tuan Chena. Omawiany tom zawiera 6 rozdziałów: 1. Morfogeneza Protozoa (V. Tartar); 2. Replikacja i funkcja jądra pierwotniaków (D. M. Prescott i G. E. Stone); 3. Rozmnażanie płciowe pierwotniaków (K. G. Grell); 4. Ekologia pierwotniaków wolnożyjących (L. E. Noland i M. Gojdics); 5. Wpływ promieniowania na Protozoa (A. C. Giese) i 6. Wzrost i rozwój pierwotniaków pasożytniczych w hodowli tkankowej (W. Trager i S. M. Krassner). Wymienione rozdziały wzbudzą z pewnością zaciekawienie nie tylko protistologów, cytofizjologów i biochemików, lecz także lekarzy interesujących się pierwotniakami pasożytniczymi, których hodowli tkankowej poświęcona jest ostatnia część książki. We współczesnej parazytologii wzrasta ogromnie zapotrzebowanie na metody hodowli pasożytów poza ustrojem żywiciela. Dobre opanowanie tych metod otwiera szeroko drogę do poznania warunków niezbędnych do osiedlenia się i rozwoju pasożyta w żywicielu, pozwoli zaznajomić się z wielu nieznanymi dotychczas i trudnymi do zbadania aspektami fizjologii układu pasożyt - żywiciel. Hodowla tkankowa nie budzi wprawdzie tak wielkich nadziei jak hodowla akseniczna, której wielką zaletą jest możność dokładniejszego zdeterminowania środowiska bytu pasożyta, lecz także stanowi cenny krok naprzód w badaniach protoparazytologicznych. W opracowaniu W. Tragera i S. M. Krassnera omówione zostały metody hodowli tkankowej pierwotniaków pasożytniczych pozakomórkowych jak *Entamoeba histolytica*, *Trichomonas gallinae*, *Trypanosoma cruzi* oraz wewnątrzkomórkowych, np. *Leishmania donovani*, różnych gatunków z rodzaju *Plasmodium*, *Eimeria acervulina i E. tenella*, *Piroplasmidae* (*Babesia* i *Theileria*), Microsporidia (*Nosema bombycis*) i wreszcie *Toxoplasma gondii*. Jak wynika z zestawienia wymienionych gatunków, stanowią one przedmiot zainteresowań parazytologii lekarskiej i weterynaryjnej. Najobszerniejszy jest rozdział referujący 69 prac szczegółowych poświęconych replikacji i funkcji jądra pierwotniaków i opublikowanych przeważnie w ostatnim 10-leciu. Omawia on trzy grupy zagadnień: syntezę kwasu dezoksyrybonukleinowego, białek i kwasu rybonukleinowego.

Książka ilustrowana jest dobrymi fotografiami oraz przejrzystymi rysunkami i wykresami.

Bogdan Czapliński

N. M. Emanuel' and Yu. N. Lyaskovskaya: THE INHIBITION OF FAT OXIDATION PROCESSES. Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig, 1967; str. 389; cena £ 1.6.0

Książka jest tłumaczeniem z języka rosyjskiego dokonanym przez K. A. Allena.

Hamowanie tlenowego rozkładu tłuszczów pokarmowych jest jednym z głównych problemów przemysłu żywieniowego. Substancje opóźniające procesy utleniania mają znaczenie także w stabilizowaniu smarów, produktów krakingu ropy naftowej, w zapobieganiu starzenia się tworzyw sztucznych i kauczuku. Autorzy w oparciu o badania własne oraz bogatą literaturę światową doko-

nali wyczerpującego przeglądu problemów zarówno teoretycznych, jak i doświadczalnych dotyczących procesów utleniania tłuszczów. Główne problemy omówiono w następujących rozdziałach: Kinetyka i chemia procesów utleniania tłuszczów i układów modelowych; Kataliza tlenowego rozkładu tłuszczów; Teoria łańcuchowa i chemia działania hamującego; Antyoxydanty i synergetyki, własności i działanie hamujące.

Książka winna zainteresować przede wszystkim pracowników przemysłu tłuszczowego i dziedzin pokrewnych. Biochemicy i toksykolodzy znajdą tu także szereg interesujących informacji.

Ryszard Niemiro

L. T. Threadgold: THE ULTRASTRUCTURE OF THE ANIMAL CELL. International Series of Monographs in Pure and Applied Biology (Zoology Division), vol. 37. Pergamon Press, Oxford 1967; str. 313, cena £ 5.—

Książka Threadgolda jest podręcznikowym opracowaniem budowy komórki zwierzęcej opartym na badaniach mikroskopowo-elektronowych. Charakter podręcznikowy tej książki podkreślają małe wprowadzające rozdziały rozrzucone w tekście, a omawiające zasady mikroskopu elektronowego, czy opisujące budowę komórki znaną z wyników badań innych niż mikroskopia elektronowa. Takim jest też rozdział wprowadzający w zagadnienia podziału komórkowego i mejozy.

Najcenniejsze oczywiście są rozdziały omawiające ultrastrukturę poszczególnych narządów. Rozdziały te są znakomicie ilustrowane materiałem fotograficznym oraz świetnymi, jakby odręcznie rysowanymi schematami. W tekście dyskutowana jest nie tylko budowa, ale i powiązania funkcjonalne omawianych struktur oraz dostępne współcześnie dane biochemiczne.

Dziewięć rozdziałów książki rozdzielono na dwie części. W pierwszej części, zawierającej 6 rozdziałów, omawiana jest budowa, a także i funkcja komórki w interfazie. Ta część kończy się omówieniem zmian w ultrastrukturze komórki w przebiegu rozwoju embrionalnego organizmu i w przebiegu różnicowania się komórki.

Druga część książki, zawierająca trzy rozdziały, poświęcona jest omówieniu ultrastruktury komórki dzielącej się. Należy podkreślić, że jest to nowatorsko i syntetycznie napisane opracowanie współczesnych poglądów na zjawiska występujące w czasie kariokinezy i mejozy.

Książkę można polecić bardzo szerokiemu gronu czytelników. Jest to podręcznik dla studentów biologii, biochemii czy medycyny, ale także cenna monografia dla pracowników naukowych zajmujących się cytofizjologią. Książka ta stanowi znakomite wprowadzenie dla tych, którzy zamierzają poświęcić się badaniom ultrastruktury materii żywej przy pomocy mikroskopu elektronowego.

Kazimierz Ostrowski

Ryszard Szepke: RADIOMETRIA STOSOWANA. Wydawnictwo Naukowo-Techniczne, Warszawa 1967; stron 234, cena 24.— zł.

W końcu ubiegłego roku ukazała się szczególnie pożyteczna książka dla lekarzy, biologów, chemików, którzy w swojej pracy stosują lub chcieliby stosować metody izotopowe. Mogą również z niej korzystać studenci wyższych uczelni, którzy pragnęliby zapoznać się lub pogłębić swoją wiedzę o technice pomiarów promieniowania jonizującego.

W książce tej, starannie wydanej przez WNT, omówiono wybrane zagadnienia z fizyki promieniowania jądrowego, detektorów promieni jonizujących, elektroniki jądrowej i statystyki pomiarowej. Bardziej szczegółowo omówiono niektóre konwencjonalne już dziś metody radiometryczne, jak pomiary aktywności beta licznikiem Geigera-Müllera, pomiary dawek promieni gamma komorą jonizującą oraz pomiary licznikiem scyntylacyjnym aktywności alfa, beta i gamma. Dokonano

też wprowadzenia do niektórych specjalnych metod, jak spektrometrii gamma, radiografii, pomiarów słabych aktywności, pomiarów promieniowania o małej energii oraz technik bezwzględnych oznaczania aktywności.

Książka zakończona została uwagami o ochronie przed promieniowaniem oraz nader cennymi w pracy laboratoryjnej i klinicznej uzupełnieniami w postaci 24 tablic z danymi liczbowymi i wykresów w dużej skali roboczej. Do każdego rozdziału podano literaturę dostępną w kraju. Książka napisana jest językiem przystępnym dla nie-fizyków z omijaniem teoretycznych wywodów, natomiast bogata jest w praktyczne wskazówki, wzory, wykresy, przykłady liczbowe itp., ułatwiające studiowanie tego bądź co bądź trudnego dla lekarzy i biologów materiału.

Mimo niewątpliwie dużego nakładu trudu ze strony autora i wydawnictwa przy wydaniu tej książki, wydaje się, że niektóre podrozdziały zostały potraktowane zbyt skromnie, jak np. dotyczące techniki pomiarów licznikami scyntylacyjnymi i licznikami proporcjonalnymi. Jeszcze mniej informacji zawiera książka o pomiarach neutronowych, gdyż właściwie ogranicza się do opisu licznika neutronów. Wydaje się również, że lekarzy i biologów zainteresowałaby wprowadzona od niedawna nowa technika pomiarów dawek metodą termoluminescencji. Autor w przedmowie wyraźnie jednak zastrzega się, że książka zawiera tylko wybrane metody, które jego zdaniem są lub będą najczęściej stosowane.

Jerzy Kawiak