ACTA
BIOCHIMICA POLONICA

QUARTERLY

Vol. 16
No. 2

W. HENDRICH

PHOTOREDUCTION OF CHLOROPHYLL b
IN THE PRESENCE OF BENZYLAMINE

Department of Biochemistry, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, ul. Chalubińskiego 4, Wrocław

The photoreduction of chlorophyll b and some of its derivatives, in benzene in the presence of benzylamine and phenylhydrazine as electron donor, was studied. Photoreduction of chlorophyll b was irreversible, and led to a mixture of products. Chlorophyll b-3-methanol underwent reversible photoreduction to 5,6-dihydrochlorophyll b-3-methanol. The reduction of magnesium-free derivatives of chlorophyll b led to formation of β,δ-dihydro compounds; in the case of pheophytin b, not only the porphyrin ring but also the aldehyde group was reduced.

Chlorophyll a and some of its derivatives participate directly in the energy conversion of photosynthesis. One of the proposed mechanisms of the action of chlorophyll at the light phase of photosynthesis consists in a reversible, redox-type reaction of the excited chlorophyll and a suitable partner. The reversible reactions of photooxidation and photoreduction of chlorophyll *in vitro* were studied as a model of this mechanism.

Photoreduction of chlorophyll a leads, depending on the conditions applied, to formation of β,δ-dihydrochlorophyll a with the absorption maximum at 525 mμ (Krasnovsky, 1948), 1,2-dihydromesochlorophyll a with the absorption maximum at 632 mμ (Seely, 1966), or 5,6-dihydrochlorophyll a (Hendrich, 1968a,b). Only the first and third reaction are reversible, and by oxidation chlorophyll a can easily be regenerated.

In the case of chlorophyll b, the experiments so far carried out have demonstrated that the photoreduction is not so easily reversible. Krasnovsky, Brin & Vojnovskaya (1949) reported that chlorophyll b was irreversibly reduced by ascorbic acid in pyridine. The obtained product, when oxidized, showed absorption maxima at 693 and 432 mμ. Reversible photoreduction of chlorophyll b was found to occur in toluene, in the presence of phenylhydrazine as electron donor (Evstigneev & Gavrilova, 1953); as a result of the reduction two products were formed, with maxima at 635 and 565 mμ. Seely (1966) studied photoreduction of chlorophyll b in a pyridine - ethanol (92:8, v/v) mixture in the presence of ascorbic acid, and found

that the Soret band of the product was shifted from 472 to 446 mµ. This could suggest the reduction of the aldehyde group at $C_{(3)}$, re-oxidation, however, led to the appearance of a compound with maxima at 642 and 440 mµ, which was not identified. On the other hand, chlorophyll b-3-methanol under similar conditions in the presence of b-1,4-diaza-bicyclo-(2,2,2)-octane was reduced to the appropriate hypochlorophyll derivative (maximum at 633 mµ), which on oxidation gave probably mesochlorophyll b-3-methanol (648 mµ in ethyl ether).

The results of experiments on photoreduction of chlorophyll b, chlorophyll b-3-methanol and their Mg-free derivatives (Fig. 1) in the presence of benzylamine are described below. Earlier studies (Hendrich, 1968a,b) have demonstrated that
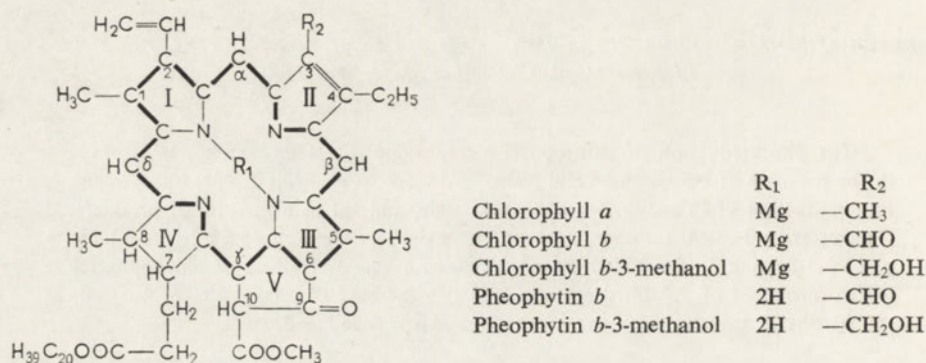

	R_1	R_2
Chlorophyll a	Mg	—CH$_3$
Chlorophyll b	Mg	—CHO
Chlorophyll b-3-methanol	Mg	—CH$_2$OH
Pheophytin b	2H	—CHO
Pheophytin b-3-methanol	2H	—CH$_2$OH

Fig. 1. Formulae of the compounds studied.

under the same conditions chlorophyll a is reversibly reduced to 5,6-dihydrochlorophyll a. It was the aim of the present work to determine whether photoreduction of chlorophyll b is also reversible, and to investigate the effect of the aldehyde group at $C_{(3)}$ on the reaction studied.

EXPERIMENTAL

Chlorophyll b was prepared from fresh spinach leaves by the method of Zscheile & Comar (1941).

Pheophytin b was prepared from chlorophyll b in the following way: to 60 ml. of ethanol containing 10 mg. of chlorophyll, 40 ml. of 2 N-HCl was added. The solution was placed in a refrigerator for 24 hr., then the crystals formed were separated by filtration. The crude preparation was purified by chromatography on a sucrose column, the pigments being eluted with a mixture of light petroleum - ethyl ether (9:2, v/v). The spectrum of the purified preparation in ethyl ether was in agreement with the published data (Goedheer, 1966).

Chlorophyll b-3-methanol was prepared by reducing chlorophyll b with sodium borohydride (Holt, 1959). The obtained crude product was transferred to ethyl ether, washed with saturated aqueous solution of NaCl and then with water. The

ether solution was dried over Na_2SO_4, evaporated to dryness under vacuum, dissolved in a mixture of light petroleum - chloroform (7:3, v/v), applied to a column of sucrose, and chromatographed with a mixture of chloroform - isopropanol - light petroleum (4:1:95, by vol.). The spectrum of the obtained preparation was in agreement with the spectrum reported by Holt (1959) for Mg-methylpheophorbide b-3--methanol.

Pheophytin b-3-methanol was obtained by treating the methanolic solution of chlorophyll b-3-methanol with 2 N-HCl. The spectrum of the obtained product in ethyl ether showed absorption bands at 662, 604, 555, 535, 506 and 412 mµ.

Menadione for biochemical use, phenylhydrazine and Kieselgel nach Stahl were products of E. Merck A. G. (Darmstadt, German Federal Republic). Other reagents were of A. R. grade, purchased from P.P.H. Polskie Odczynniki Chemiczne (Gliwice, Poland). Light petroleum was purified by shaking with conc. H_2SO_4, sat. $KMnO_4$ in 10% H_2SO_4, washing with water, drying over $CaCl_2$ and distilling over sodium. The fraction 40 - 60° was used. Benzene was distilled over sodium.

The instruments and the experimental procedure applied were as described in the previous work (Hendrich, 1968a). Schott (Jena, German Democratic Republic) filters were used throughout.

RESULTS

Chlorophyll b

As it could be expected from the data published by other authors, photoreduction of chlorophyll b in the presence of benzylamine appeared to be a more complex process than photoreduction of chlorophyll a. The reaction was carried out in benzene in the presence of 4 - 10% of benzylamine and 3 - 10 mM-phenylhydrazine. Illumination with red light (RG2 filter) of the de-aerated solution caused a large decrease of the main absorption band of chlorophyll b at 647 mµ with simultaneous appearance of bands at about 635 and 611 mµ, and a weak band at 565 mµ (Fig. 2). The photoreduction products were partly separated by chromatography. Solutions of the pigments in light petroleum were applied to the sucrose column and eluted with chloroform - light petroleum (1:9, v/v). Three fractions were obtained: a green, a violet and a green-yellow. The lowest green-yellow fraction was not oxidized either by oxygen or menadione. Its spectrum in ethyl ether (maxima at 657 and 428 mµ) and thin-layer chromatography on Kieselgel (with ethyl ether - light petroleum - chloroform, 5:4:2, by vol., as solvent) indicated that chlorophyll b-3-methanol was the main component of this fraction. The other two fractions, the violet and the green one, were photooxidized in the presence of menadione and pyridine. The violet fraction showing absorption bands at 603, 417 and 400 mµ was photochemically oxidized (OG3 filter) by menadione in benzene solution in the presence of pyridine, yielding chlorophyll b-3-methanol as the main product (Fig. 3). On the other hand, the green fraction contained some compounds which we were unable
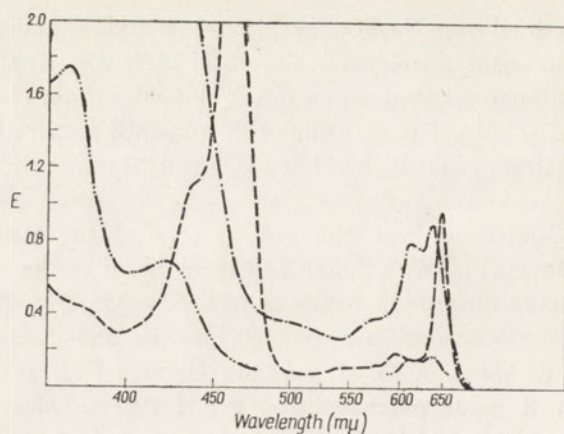
Fig. 2. Spectrum of: (———), starting solution of 0.13 mM-chlorophyll b in benzene with the addition of 4% benzylamine and 3 mM-phenylhydrazine, and the total diluted with benzene at a ratio of 1:5; (—·—), starting solution after illumination for 60 min. with red light (RG2 filter) and diluted with benzene at a ratio of 1:4; (—··—), the same illuminated solution diluted with benzene at a ratio of 1:20.
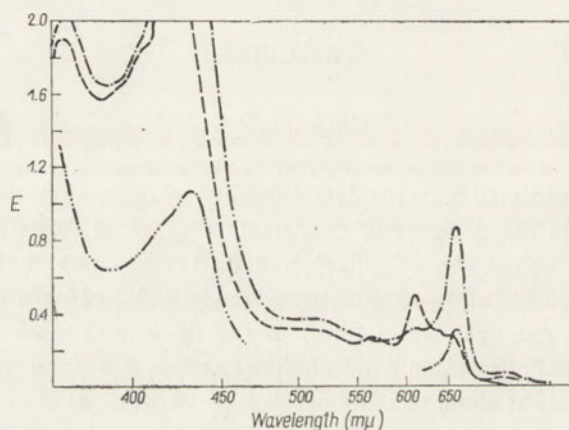

Fig. 3. Spectrum of: (———) the violet fraction of the photoreduction product of chlorophyll b in benzene with the addition of pyridine (2%) and menadione (1 mg./ml.); (—·—), the solution after 20 min. illumination with orange light (filter OG3); (—··—), the same illuminated solution after dilution with benzene at a ratio of 1:3.

to separate by column chromatography using the following solvents: ethyl ether - light petroleum, 1:1 and 1:5 (v/v); 5 - 20% chloroform in light petroleum; 0.5 - 1.5% n-propanol or isopropanol in light petroleum. After photooxidation (OG3 filter) with menadione or 2,6-dichlorophenol indophenol in benzene with the addition of 2% pyridine, a mixture of products was obtained showing an asymmetrical absorption band at about 657 mμ. Thin-layer chromatography on Kieselgel (with ethyl ether - light petroleum - chloroform, 5:4:2, by vol., as solvent) demonstrated

the presence of pheophytin b, chlorophyll b-3-methanol, chlorophyll b and an aminolysis product of chlorophyll b.

The direction of the photoreduction was to some extent influenced by the wavelength of the exciting light. On illumination through interference filters with maximum transmission at 661 and 643 mμ, the main photoreduction product had the maximum at 635 mμ. On the other hand, when an interference filter with maximum transmission at 622 mμ was used, the photoreduction was very slow due to low light absorption by the solution at this wavelength region, and, along with the product with the maximum at 635 mμ, a compound with the maximum at 611 mμ was obtained.

When in the reaction mixture the content of water or ethanol was increased, the photoreduction gave as the main product the compound with the maximum at 635 mμ, which did not undergo re-oxidation; on the basis of its spectrum and thin-layer chromatography, it was identified as an aminolysis product of chlorophyll b.

Chlorophyll b-3-methanol

From the above-described experiments it follows that in the presence of benzylamine both the porphyrin ring and the aldehyde group undergo reduction. Therefore it could be expected that in the presence of benzylamine the photoreduction of chlorophyll b-3-methanol in which the aldehyde group was reduced to an alcohol group,
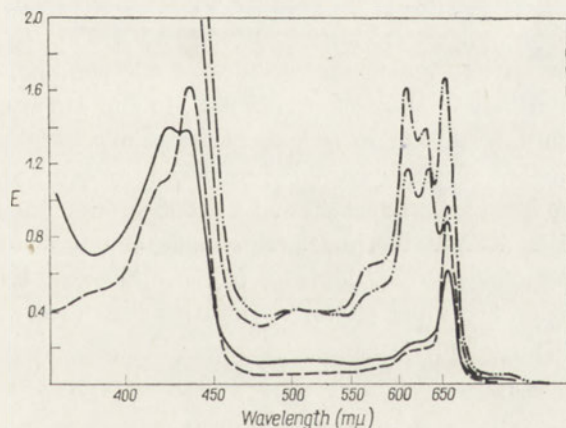
Fig. 4. Spectrum of: (— — —), starting solution of chlorophyll b-3-methanol in benzene (diluted with benzene at ratio of 1:4); (—·—), starting solution after addition of 4% benzylamine, 3mM--phenylhydrazine, de-aeration and illumination for 60 min. with red light (RG2 filter); (—··—), the above solution after removal of benzylamine and phenylhydrazine, addition of 2% pyridine and menadione (1 mg./ml.), de-aeration and illumination for 10 min. with orange light (OG3 filter); (———), the above solution after further illumination for 30 min. and dilution with benzene at a ratio of 1:4. The bands at 660 and 433 mμ correspond to the regenerated chlorophyll b-3-methanol, the band at 422 mμ and the weak band at 632 mμ correspond to the aminolysis product of chlorophyll b-3-methanol.

would be reversible. This in fact proved to be the case. A solution of chlorophyll
b-3-methanol and phenylhydrazine in benzene containing 4 - 10% of benzylamine
was reduced on illumination with red light (RG2 filter) to a product with the maxi-
mum at 611 mμ, the reaction being complete after 60 min. illumination. The main
product was accompanied by a by-product with the maximum at 632 mμ, which
probably was an aminolysis product of chlorophyll b-3-methanol. The main pro-
duct was not oxidized by oxygen, but in a benzene or ether solution containing 2%
of pyridine it underwent photochemical oxidation (OG3 filter) by menadione or
2,6-dichlorophenol indophenol to chlorophyll b-3-methanol, which was identified
both on the basis of its spectrum and its ability to be photoreduced again to the
product with the maximum at 611 mμ. Changes in the spectrum due to photore-
duction and re-oxidation of chlorophyll b-3-methanol are shown in Fig. 4.

Pheophytin b *and pheophytin* b-*3-methanol*

Experiments on chlorophyll *a* (Hendrich, 1968a,b) demonstrated that the
presence of Mg atom in the molecule of substrate was necessary to direct the
photoreduction in the presence of benzylamine toward the 5,6-dihydroderivatives.
The aim of the present series of experiments was to check whether the Mg atom
plays a similar role also in chlorophyll *b*.

The de-aerated solution of pheophytin *b* and phenylhydrazine in benzene con-
taining 4% of benzylamine turned red-brown after 15 min. illumination through
a red filter (RG2). The solution showed a broad, flat absorption band at 525 - 505 mμ
and the Soret band at 410 mμ. This product was oxidized in darkness by atmo-
spheric oxygen to pheophytin *b*-3-methanol (664 and 410 mμ in benzene). When
photoreduction was carried out in the presence of 4% pyridine, the product was
red and its spectrum showed absorption bands at 515 and 415 mμ. This compound
was oxidized in darkness by air oxygen to pheophytin *b*.

On photoreduction of pheophytin *b*-3-methanol in benzene containing 4%
of benzylamine, the obtained product showed a flat absorption band at 520 - 505 mμ
and the Soret band at 408 mμ. Its spectrum resembled closely that of the compound
obtained by photoreduction of pheophytin *b*, the differences being possibly due
to by-products. In darkness the photoreduction product underwent oxidation by
oxygen to pheophytin *b*-3-methanol.

DISCUSSION

Of the compounds submitted to photoreduction in the presence of benzylamine,
chlorophyll b-3-methanol showed the greatest similarity to chlorophyll *a*. The
photochemical reduction led to formation of a product with the maximum at 611 mμ,
which probably was 5,6-dihydrochlorophyll b-3-methanol and which underwent
photochemical oxidation to the starting compound. These results are not surprising.
Replacement of the $-CH_3$ group in chlorophyll *a* by $-CH_2OH$ in chlorophyll
b-3-methanol should lead neither to greater changes in chemical properties nor

to marked changes in the distribution of the density of the electron cloud in the porphyrin ring (as evidenced e.g. by the marked similarity of the absorption spectra) so that the photoreduction of these two compounds could be expected to have a similar course.

Chlorophyll *b* behaves in a different manner. The reduction of the porphyrin ring occurs in the presence of benzylamine at the same position as in the presence of pyridine, and products with absorption bands at 635 and 565 mµ are formed. The specificity of action of benzylamine manifests itself only in the additional reduction of the aldehyde group, and formation of the compound with the maximum at 611 mµ, which on oxidation gives chlorophyll *b*-3-methanol. Presumably the presence of the double bond $C=O$ at $C_{(3)}$, conjugated with the porphyrin ring, prevents either the formation of the appropriate complex of chlorophyll with benzylamine (Hendrich, 1967) or the isomerization of the initial photoreduction product to the 5,6-dihydroderivative (Seely, 1966). After saturation of this double bond, the interfering effect disappears and photoreduction leads to a product specific for the action of the amine.

It seems also of interest that the ability of benzylamine to direct the reaction toward reduction of the aldehyde group does not depend on the presence of the Mg atom. Photoreduction of pheophytin *b* in the presence of benzylamine gives a product which is oxidized to pheophytin *b*-3-methanol. The action of this type was not observed with pyridine: in its presence the photoreduction of pheophytin *b* gives a product from which pheophytin *b*, and not pheophytin *b*-3-methanol, is regenerated.

Both the spectrum of the photoreduction product of pheophytin *b*-3-methanol in the presence of benzylamine (absorption band at 520 - 505 mµ) and its ability to undergo oxidation by oxygen in darkness, indicate that the porphyrin ring of this compound is reduced at positions β and δ, and not 5 and 6. Thus the directing action of benzylamine on the reduction toward formation of 5,6-dihydro compounds is for the derivatives of chlorophyll *b*, similarly as for those of chlorophyll *a*, dependent on the presence of the Mg atom.

REFERENCES

Evstigneev W. B. & Gavrilova W. A. (1953). *Dokl. Acad. Nauk SSSR* **91**, 899.

Goedheer J. C. (1966). In *The Chlorophylls* (L. P. Vernon & G. R. Seely, eds.) p. 158. Academic Press, New York.

Hendrich W. (1967). *Roczniki Chem.* **41**, 743.

Hendrich W. (1968a). *Biochim. Biophys. Acta* **162**, 265.

Hendrich W. (1968b). *Roczniki Chem.* **42**, 1715.

Holt A. S. (1959). *Plant Physiol.* **34**, 310.

Krasnovsky A. A. (1948). *Dokl. Acad. Nauk SSSR* **60**, 421.

Krasnovsky A. A., Brin G. P. & Vojnovskaya K. K. (1949). *Dokl. Acad. Nauk SSSR* **69**, 393.

Seely G. R. (1966). *J. Amer. Chem. Soc.* **88**, 3417.

Zscheile F. P. & Comar C. L. (1941). *Botan. Gaz.* **102**, 463.

FOTOREDUKCJA CHLOROFILU *b* W OBECNOŚCI BENZYLOAMINY

Streszczenie

Badano reakcję fotoredukcji chlorofilu *b* i jego pochodnych w benzenie z dodatkiem benzyloaminy i fenylohydrazyny jako donatora elektronów. Fotoredukcja chlorofilu *b* przebiega nieodwracalnie i prowadzi do mieszaniny produktów. Chlorofil-*b*-3-metanol podlega odwracalnej fotoredukcji do 5,6-dwuhydrochlorofil-*b*-3-metanolu. Redukcja bezmagnezowych pochodnych chlorofilu *b* prowadzi do β,δ-dwuhydropochodnych; w przypadku feofityny *b* redukcji podlega nie tylko pierścień porfirynowy, ale również grupa aldehydowa.

ALICJA BARDOŃ, ZOFIA PAMUŁA and M. HILLAR

ISOLATION AND PURIFICATION OF AN ALKALINE RIBONUCLEASE FROM HUMAN PLACENTA

Department of Biochemistry, Medical School, Al. Zwycięstwa 42, Gdańsk

1. An alkaline ribonuclease from human placenta was purified about 20 000-fold. 2. The purification procedure involved extraction with sulphosalicylic acid, chromatography on DEAE-cellulose, and SE-Sephadex C-25 gel filtration. 3. The obtained enzyme preparation was free of alkaline and acid phosphatases and of non-specific phosphodiesterase activities. 4. The optimum pH for the alkaline ribonuclease was 8.5.

Two types of ribonucleases have been shown to occur in animal tissues: an acid RNase, heat- and acid-labile (Maver & Greco, 1949; Zytko, De Lamirande, Allard & Cantero, 1958; Maver & Greco, 1962), and an alkaline RNase, heat--stable and extractable with dilute acids (Roth, 1957, 1958). Most preparations of alkaline RNase reported so far were purified 60 - 400-fold. However, greater purifications have also been achieved. Roth (1957) purified about 1000-fold a ribonuclease from rat-liver mitochondria; Beard & Razell (1964) described a preparation from hog-liver mitochondria and cytoplasm, which was purified about 3000--fold; Gordon (1965) obtained the enzyme with the highest reported degree of purification (6000-fold) from rat-liver homogenate.

Roth (1962) and Shortman (1962) demonstrated the occurrence in liver and other tissues of an inhibitor of alkaline ribonuclease. The inhibitor was found to be heat- and acid-labile, and to be inactivated by sulphydryl reagents.

In the present work, alkaline ribonuclease from human placenta was investigated. The presence of this enzyme in the cytoplasm of human placenta was reported by Brody (1957), and its activity was observed to decrease with age. For the purification of the enzyme we have applied sulphosalicylic acid[1] extraction as SSA had been found to extract rather specifically the proteins showing ribonuclease activity (Bardoń & Rzeczycki, 1966; Bardoń & Pamuła, 1967).

---

[1] Abbreviations: SSA, sulphosalicylic acid; PCMB, *p*-chloromercuribenzoate.

MATERIALS AND METHODS

Reagents. Yeast RNA (B.D.H., Poole, England) was purified as described previously (Bardoń & Pamuła, 1967); DEAE-cellulose DE II Whatman, nominal capacity 1.0 m-equiv./g., was produced by W. R. Balston Ltd. (England); SE-Sephadex C-25 was a product of Pharmacia (Uppsala, Sweden). Sulphosalicylic acid, EDTA and $MgSO_4$ were purchased from Biuro Obrotu Odczynnikami Chemicznymi (Gliwice, Poland). Tannic acid, a product of the New York Quinine and Chemicals Works Inc. (U.S.A.), was purified after Mejbaum-Katzenellenbogen & Kudrewicz-Hubicka (1966). Calcium salt of bis-*p*-nitrophenylphosphate was a product of Sigma Co. (St. Louis, Mo., U.S.A.), *β*-glycerolphosphate, of L. Light & Co. Ltd. (Colnbrook, England) and *p*-chloromercuribenzoic acid of B.D.H. (Poole, England).

Preparation of DEAE-cellulose. DEAE-cellulose was washed repeatedly to release the minute grains. Then it was placed in a column (17 cm. × 1.5 cm.) and rinsed with 2 l. of 0.02 M-sodium acetate buffer of pH 7.0.

Preparation of SE-Sephadex C-25. Sephadex, 10 g., was suspended in 2 l. of water and allowed to settle overnight. Then it was rinsed several times with 0.5 N-HCl (usually about 0.5 l. being used), then with water to neutral pH. Afterwards it was successively washed with 0.5 l. of 0.5 N-NaOH, again with water to neutral pH and at last with 4 l. of 0.05 M-sodium acetate buffer, pH 5.6. The Sephadex prepared in this way was applied to a column (16 cm. × 1.5 cm.) and equilibrated with 1 l. of the above buffer.

Ribonuclease activity. For the assays, the method of Roth (1962) was employed. The incubation mixtu e contained in 1 ml.: 0.7 or 0.6 ml. of twice-diluted universal Davies' (1959) buffer of pH 8.5; 0.1 ml. of 0.075 M-EDTA; and 0.1 ml. of enzyme preparation in 0.05 M-sodium acetate buffer, pH 5.0. The samples were preincubated for 3 min. at 37°, then the reaction was started by adding 1.5 mg. of yeast RNA, and after 30 min. stopped by the addition of 1 ml. of 1 N-HCl in 76% ethanol. The samples were then cooled in an ice bath for 5 min. and undigested RNA was centrifuged off. The supernatant was diluted with two volumes of watei, and acid-soluble nucleotides were determined by measuring the extinction at 260 mμ with a Unicam SP 500 spectrophotometer. The blank was prepared in the same way except that the enzyme preparation was pipetted in after the addition of acid ethanol. The enzyme activity was expressed as ΔE_{260} after 30 min. incubation.

Non-specific phosphodiesterase activity. This was measured according to Ostrowski & Walczak (1961) using nitrophenyl phosphate as substrate.

Acid and alkaline phosphatases activity. Substrates and incubation mixtures we e prepared as described by Kaser (1958). Inorganic phosphate released from glycerolphosphate was determined by the method of Gomori (1953).

Protein content. Protein in the cell-sap was determined by the biuret method (Layne, 1957), and throughout the purification procedure the tannin method of Mejbaum-Katzenellenbogen (1955) was employed.

RESULTS

Isolation of alkaline ribonuclease

Preparation of homogenate. Fresh human placentae (within two hours after the delivery) were obtained from the Obstetrics Clinic of the Medical School in Gdańsk. The tissue was sliced and washed first with water to remove blood and then with 0.25 M-sucrose solution until the washings had only a faint pinky colour. The slices were then ground in a meat grinder, suspended in 4 volumes of 0.25 M--sucrose solution and homogenized for 1 min. in a Waring blendor at 3000 rev./min. The obtained homogenate was centrifuged at 1000 g for 10 min., the sediment discarded and the supernatant, consisting of ribosomes, mitochondria and cyto-plasm, was used in the purification procedure. This preparation is further referred to as cell-sap.

Extraction with sulphosalicylic acid. To 9 volumes of the cell-sap, 1 volume of 2 M-SSA was added with continuous stirring. After 30 min. the precipitate formed was centrifuged off at 1000 g for 10 min. and the supernatant adjusted with 4 M--NaOH to pH 5.0. Usually the supernatant was clear; if not, it was filtered through Whatman no. 1 paper.
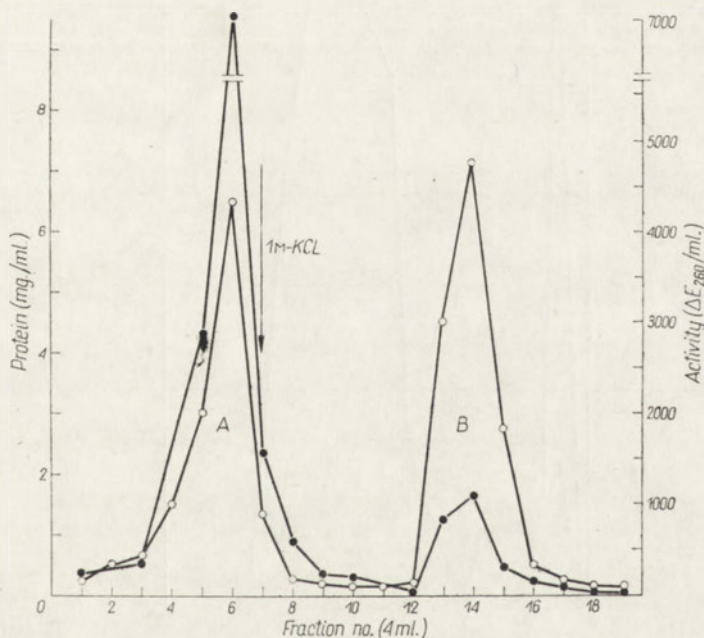
Fig. 1. DEAE-cellulose column chromatography of the proteins extracted with SSA from the cell--sap of human placenta and concentrated by the tannin-caffeine procedure. To the column (17 × ×1.5 cm.), 110 mg. of protein in 5 ml. of acetate buffer, pH 5.0, was applied and eluted first with a sodium acetate concentration gradient (0.02 - 0.04 M), the pH of the effluent decreasing from pH 7.0 to 5.3 (the mixing vessel contained 200 ml. of 0.02 M-sodium acetate buffer, pH 7.0, and the reservoir 0.2 M-acetate buffer, pH 5.0), and then with 1 M-KCl in 0.04 M-sodium acetate buffer, pH 5.3. Fractions of 4 ml. were collected. (○), Protein; (●), Ribonuclease activity.

Concentration of protein. To concentrate the proteins from the SSA-extract, the tannin-caffeine procedure of Mejbaum-Katzenellenbogen (1959) was employed with some modifications. The proteins were precipitated with tannin added at a ratio of 1:50 (w/w), and after 30 min. the tannin-protein complex was centrifuged at 20 000 g for 10 min. Then the proteins were regenerated from the complex by the addition of caffeine *in substantia* at a protein to caffeine weight ratio of 1:2. The mixture was mixed thoroughly and allowed to stand overnight at 4°. Then 0.02 M--sodium acetate buffer, pH 5.0, was added in such an amount as to obtain a 2 - 2.5% solution of the regenerated proteins. The formed tannin-caffeine complex was centrifuged off at 20 000 g for 10 min., and the supernatant submitted to column chromatography.

DEAE-cellulose chromatography. From the DEAE-cellulose, the protein was eluted as described by Maver & Greco (1962); a sodium acetate linear concentration gradient from 0.02 to 0.04 M was used, and the pH of the effluent decreased con-
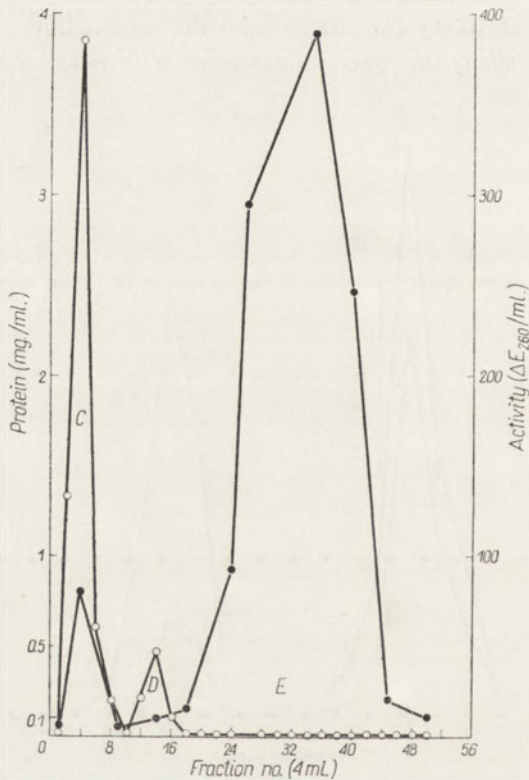
Fig. 2. SE-Sephadex C-25 gel filtration of fraction *A* from DEAE-cellulose chromatography. To the column (16 × 1.5 cm.), 40 mg. of protein was applied (5 ml.) and eluted with a KCl concentration gradient (up to 0.6 M) in sodium acetate buffer, the pH of the effluent decreasing from pH 5.6 to 4.4. The mixing vessel contained 150 ml. of 0.05 M-acetate buffer, pH 5.6, and the reservoir 0.6 M-KCl in 0.05 M-acetate buffer, pH 4.4. Fractions of 4 ml. were collected. (○), Protein; (●), ribonuclease activity.

comitantly from pH 7.0 to 5.3. Then 1 M-KCl in 0.04 M-sodium acetate buffer, pH 5.3, was applied. The elution pattern is shown in Fig. 1. Of the two fractions obtained, fraction *A*, eluted with the sodium acetate concentration gradient, exhibited ribonuclease activity six times as high as that of fraction *B* which was eluted with KCl.

SE-Sephadex C-25 gel filtration. The DEAE-cellulose fraction *A* was resolved by gel filtration on SE-Sephadex C-25 column into three fractions, of which fraction *E* was active, and fractions *C* and *D* were inactive (Fig. 2).

Table 1

Purification of alkaline ribonuclease from human placenta

Starting material consisted of 5 human placentae (about 1500 g. of fresh tissue). The activity was determined as described in Methods and expressed as $\Delta E_{260}/30$ min. incubation. For details see text.

Purification step	Total protein (mg.)	Total activity (ΔE_{260})	Specific activity (ΔE_{260}/mg. protein)	Purification factor	Yield (%)
Cell-sap	20 985	75 546*	3.6*	—	100
0.2 M-SSA-extract	155	60 450	390	108	71
Protein concentrated by the tannin-caffeine procedure	110	48 400	440	122	64
DEAE-cellulose fraction *A*	40	44 000	1 100	305	58
SE-Sephadex C-25 fraction *E*	0.343	24 700	72 000	20 000	32

* The activity was determined in the presence of 0.001 M-PCMB. Without PCMB added the specific activity amounted to only 0.53 ΔE_{260}/mg. protein.

A summary of the purification procedure is presented in Table 1. Extraction with 0.2 M-SSA, chromatography on DEAE-cellulose and gel filtration on SE--Sephadex gave 100-, 300-, and 20 000-fold purification, respectively. The purified enzyme showed no acid or alkaline phosphatase, nor non-specific phosphodiesterase activity.

Some properties of the enzyme

Similarly to what has been demonstrated for various animal tissues, also the placenta contains a natural inhibitor of alkaline RNase. The enzyme activity in crude cell-sap proved to be rather small, about 0.45 ΔE_{260}/mg. protein, whereas the addition of PCMB to 0.001 M concentration resulted in a 7 - 10-fold activation of the RNase (Table 2). In the SSA-extracts total activity was present irrespective of the addition of PCMB, showing that the inhibitor was not extracted with SSA or was destroyed by the acid.

Table 2

*Effect of PCMB on the alkaline ribonuclease activity in placental cell-sap and in SSA-
-extract*

The activity was assayed as described in Methods. Mean values from three determinations are
given, with limit values in parentheses.

Addition	Cell-sap	SSA-extract
	Specific activity (ΔE_{260}/mg. protein)	
None	0.45 (0.39 - 0.53)	346 (310 - 390)
PCMB, 0.001 M	3.7 (3.6 - 4.4)	346 (310 - 390)

Table 3

Effect of time of heating on alkaline ribonuclease activity

The solution of the purified enzyme preparation in sodium acetate buffer of pH 5.0 was heated in
a boiling-water bath, then cooled, and the enzyme activity was determined at pH 8.5. The results
are expressed as percentages of the activity of the non-heated sample.

Time of heating (min.)	Activity (%)
—	100
5	70
10	55

Table 4

Effect of Mg^{2+} ion on activity of the purified alkaline ribonuclease preparation

Enzyme activity is expressed as percentage of the activity without Mg^{2+} added. The activity was
measured as described in Methods except that EDTA was omitted.

Mg^{2+} added	Activity (%)
None	100
0.005 M	100
0.05 M	75
0.1 M	60

If the degree of purification of our placental RNase preparation is calculated
in relation to the activity of the crude extract measured in the absence of PCMB,
then it appears that the obtained preparation was purified about 136 000-fold.

To characterize the enzyme, the optimum pH, the effect of heating and that
of Mg^{2+} ions were investigated. The purified enzyme exhibited one pH maximum
at 8.5 (Fig. 3). However, for the cell-sap to which no PCMB was added, two optima
were found: at pH 6.0 (for acid ribonuclease unextractable with acids), and at
pH 9.5 (Fig. 3). The difference in pH optimum for the enzyme activity in alkaline
medium may indicate that in the crude preparation at pH 9.5 the inhibitor was
less active than at lower pH values. The purified enzyme heated at pH 5.0 in a boiling-
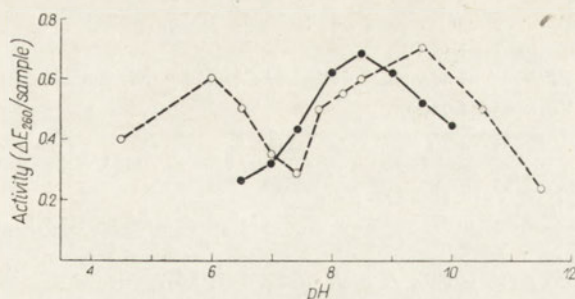
Fig. 3. Effect of pH on the ribonuclease activity of ($\circ$), placental cell-sap, and ($\bullet$), the purified preparation. Twice diluted universal Davies' buffer was used.

Table 5

Comparison of the alkaline ribonuclease activity in the SSA- and H_2SO_4-extracts

The SSA-extract was prepared as described in the text. To obtain the H_2SO_4-extract, 1 volume of 2.5 N-H_2SO_4 was added with stirring to 9 volumes of placental cell-sap. After 30 min. the mixture was neutralized to pH 7.0 and filtered through Whatman no. 3 paper. The activity was determined as described in Methods.

	Cell-sap	SSA-extract	H_2SO_4-extract
Protein, mg.	2100	15.3	365
Total activity, ΔE_{260}	7560*	5970	6570
Specific activity,			
ΔE_{260}/mg. protein	3.6*	390	18
Purification factor	—	108	5

* The activity was determined in the presence of 0.001 M-PCMB.

-water bath for 5 and 10 min. lost respectively 30 and 40% of its activity (Table 3). Similarly to the results reported by Roth (1954), our preparation was inhibited by high concentrations of Mg^{2+} ion to a small extent only (Table 4).

For extraction of the alkaline ribonuclease from the tissue we employed 0.2 M-SSA instead of the commonly used 0 25 M-H_2SO_4. The comparison of the two extraction procedures (Table 5) showed that the extracted activity was in both cases practically the same, but the SSA-extract contained 25 times less protein so that the purification factor obtained with SSA was about 20 times higher than in the case of H_2SO_4 extraction.

REFERENCES

Bardoń A. & Pamuła Z. (1967). *Acta Biochim. Polon.* **14**, 341.
Bardoń A. & Rzeczycki W. (1966). *Acta Biochim. Polon.* **13**, 218.
Beard J. R. & Razell W. E. (1964). *J. Biol. Chem.* **239**, 4186.

Brody S. (1957). *Biochim. Biophys. Acta* **24**, 502.

Davies M. T. (1959). *Analyst* **84**, 197.

Gomori G. (1953). In *Standard Methods of Clinical Chemistry* (M. Reiner, ed.) vol. 1, p. 84. Acad. Press, New York and London.

Gordon J. (1965). *Arch. Biochem. Biophys.* **112**, 421.

Kaser M. M. (1958). In *Standard Methods of Clinical Chemistry* (D. Seligson, ed.) vol. 2, p. 122. Acad. Press, New York and London.

Layne R. (1957). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 450. Acad. Press, New York.

Maver M. E. & Greco A. E. (1949). *J. Biol. Chem.* **181**, 861.

Maver M. E. & Greco A. E. (1962). *J. Biol. Chem.* **237**, 736.

Mejbaum-Katzenellenbogen W. (1955). *Acta Biochim. Polon.* **2**, 279.

Mejbaum-Katzenellenbogen W. (1959). *Acta Biochim. Polon.* **6**, 385.

Mejbaum-Katzenellenbogen W. & Kudrewicz-Hubicka Z. (1966). *Acta Biochim. Polon.* **13**, 57.

Ostrowski W. & Walczak Z. (1961). *Acta Biochim. Polon.* **8**, 345.

Roth J. S. (1954). *J. Biol. Chem.* **208**, 181.

Roth J. S. (1957). *J. Biol. Chem.* **227**, 591.

Roth J. S. (1958). *J. Biol. Chem.* **231**, 1097.

Roth J. S. (1962). *Biochim. Biophys. Acta* **61**, 903.

Shortman K. (1962). *Biochim. Biophys. Acta* **55**, 88.

Zytko J., De Lamirande G., Allard C. & Cantero A. (1958). *Biochim. Biophys. Acta* **27**, 495.

IZOLACJA I OCZYSZCZANIE ALKALICZNEJ RYBONUKLEAZY Z ŁOŻYSKA LUDZKIEGO

Streszczenie

1. Alkaliczną rybonukleazę z łożyska ludzkiego oczyszczono około 20 000 razy.

2. Procedura oczyszczania polegała na ekstrakcji cytoplazmy kwasem sulfosalicylowym, chromatografii na DEAE-celulozie i sączeniu na SE-Sephadex C-25.

3. Preparat nie wykazywał aktywności niespecyficznej fosfodwuesterazy, ani alkalicznej i kwaśnej fosfatazy.

4. Optimum pH enzymu wynosiło 8,5.

DANUTA HULANICKA, T. KŁOPOTOWSKI and G. BAGDASARIAN

INHIBITION OF AMINOIMIDAZOLE RIBOTIDE BIOSYNTHESIS
IN *SALMONELLA TYPHIMURIUM* BY AMINOTRIAZOLE

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, Warszawa 12

Experiments on purineless mutants of *Salmonella typhimurium* suggest that aminotriazole inhibits the reaction of cyclization of formylglycineamidine ribotide to aminoimidazole ribotide.

Aminotriazole[1] is a competitive inhibitor of imidazoleglycerolphosphate dehydratase (EC 4.2.1.19), an enzyme of histidine biosynthesis, present in bacteria, yeast and higher plants (Hilton, Kearney & Ames, 1965; Kłopotowski & Wiater, 1965; Wiater & Krajewska, 1968). However, at higher aminotriazole concentrations histidine alone is unable to restore the growth of *Salmonella typhimurium* (Hilton *et al.*, 1965) and *Saccharomyces cerevisiae* (Kłopotowski & Bagdasarian, 1966) to the normal rate. The addition of histidine and adenine is needed to nullify completely the effect of high aminotriazole concentration both in the bacterium and the yeast. This suggested that aminotriazole inhibits in micro-organisms not only biosynthesis of histidine, but also that of purine. In this paper the evidence is presented that aminotriazole inhibits purine biosynthesis at a step not later than that of AIR formation.

MATERIALS AND METHODS

Organism. The following mutants of *S. typhimurium* were used (the names of enzymes coded for by the respective genes are given in parentheses): *purA1* (adenylosuccinate synthetase, EC 6.3.4.4.), *purB12* (adenylosuccinate lyase, EC 4.3.2.2) *purC7* (phosphoribosylaminoimidazole carboxylase, EC 4.1.1.21), *pur D11* (phosphoribosylglycineamide synthetase, EC 6.3.1.3), *purF145* (phosphoribosylpyrophosphate amidotransferase, EC 2.4.2.14), *purG310* (phosphoribosylformylglycineamidine synthetase, EC 6.3.5.3), *purI305* (phosphoribosylaminoimidazole synthetase, EC

---

[1] Abbreviations: AMP, adenosine-5'-monophosphate; AIR, aminoimidazole ribotide; aminotriazole, 3-amino-1,2,4-triazole; FGAM, formylglycineamidine ribotide; IMP, inosine-5'-monophosphate.

[127]

6.3.3.1). All these mutants were kindly supplied by Professor J. S. Gots from Philadelphia. As wild type, the strain LT-2 was used.

Media. The minimal medium C (Vogel & Bonner, 1956) was supplemented with 0.5% glucose or, to avoid the hexose interference with ribose determination by orcinol method, 0.5% sodium lactate. To grow the mutants 0.2 mM-adenine and 0.01 mM-thiamine was added to the medium.

Cultures. The bacteria were cultivated at 37° with shaking. The growth was followed turbidimetrically at 420 mμ. The extinction readings were converted to μg. dry weight using a calibration curve. Cells were harvested at the end of logarithmic phase of growth (about 400 μg. dry wt. per ml.) by centrifugation and washed three times with isotonic saline buffered with phosphate, pH 7.4.

Accumulation of intermediates of purine biosynthesis. Cells of purine mutants were shaken at 37° under non-growth conditions in an incubation mixture containing 20 mM-potassium phosphate buffer, pH 7.4, 1 mM-L-glutamine, 2 mM-DL-serine, 30 mM-sodium chloride, 30 mM-sodium lactate and mutant cells suspension, 1 mg. dry wt. per ml. After 2 hr. the incubation mixture was centrifuged and the accumulation of intermediate was determined in supernatant and in cells.

Cell-free extracts. The washed cells were resuspended in two volumes of 0.15 M-potassium phosphate buffer, pH 7.4, and disrupted in a laboratory press (Eaton, 1962). The extract was centrifuged at 15 000 g for 15 min. The sediment was extracted with the same buffer and the supernatants were combined.

Analytical determinations. Arylamine intermediates of purine biosynthesis were determined by the modified Bratton & Marshall method (Kłopotowski & Hulanicka, 1963). The concentration of sodium nitrite was 0.6% and that of ammonium sulphamate 4%. The time interval between the additions of sodium nitrite and ammonium sulphamate was 3 min. At 30 min. after the addition of ammonium sulphamate N-naphthyl-p-ethylenediamine solution was added. Aminotriazole up to 20 μmoles per assay did not yield any colour. The extinction readings at 500 mμ were converted to mμmoles of aminoimidazole compounds using extinction coefficient of 24 600 cm.^{2} mole^{-1} (Lukens & Flaks, 1963). The total ribose containing intermediates of purine biosynthesis were determined by the orcinol method of Mejbaum (Buchanan, 1956). All samples were hydrolysed in 1.3 N-hydrochloric acid at 100° for 40 min. before the colour development. Protein in cell-free extracts was determined spectrophotometrically according to the method of Warburg & Christian (Layne, 1957).

Enzyme assays. Adenylosuccinate synthetase activity was determined by following the increase of absorbance at 280 mμ (Lieberman, 1963). The incubation mixture contained in a volume of 0.7 ml.: 0.1 m-mole of glycine buffer, pH 8.0, 4 μmoles of MgCl$_2$, 0.04 μmole of ATP, 0.4 μmole of phosphoenolpyruvate, 20 units of phosphopyruvate kinase, 0.5 μmole of aspartate, 10 mμmoles of GTP, 0.3 μmole of IMP and 0.27 mg. protein of cell-free extracts of the mutant *purB12*. After 30 min. of incubation, 0.5 ml. of 6% perchloric acid was added and the insoluble material was removed by centrifugation. The increase of absorbance at 280 mμ was measured.

Adenylosuccinate lyase activity was studied by measuring the amount of succinyl-AMP formed from AMP and fumaric acid (Carter & Cohen, 1956). Cell-free extract was prepared from the mutant *purA1* and purified to remove fumarase activity. Fraction IV after heat treatment was used. The incubation mixture contained in 3.0 ml.: 9.0 µmoles of fumaric acid, 1.3 µmoles of AMP, 125 µmoles of phosphate buffer, pH 5.0, and 0.30 mg. protein of enzyme preparation. The increase in absorbance at 283 mµ was measured. The sample containing 10 mM-aminotriazole was measured against the blank with 10 mM-aminotriazole.

One unit of enzyme activity was defined as a change in absorbance of 0.001 per min.

Chemicals. The following chemicals were used: adenine (Reanal, Budapest, Hungary), adenosine, inosine, AMP, GTP, IMP, phosphoenolpyruvic acid potassium salt, pyruvate kinase (Sigma, Chem. Co., St. Louis, Mo, U.S.A.), hypoxanthine (Permedia, Lublin, Poland), ATP, DL-serine (Calbiochem, Los Angeles, Calif., U.S.A.), aminotriazole (Fluka, Buchs, Switzerland), L-histidine (Chemapol, Prague, Czechoslovakia), fumaric acid, L-aspartic acid (B.D.H., Poole, England), L-glutamine (Nutr. Biochem. Corp., Cleveland, Ohio, U.S.A).

RESULTS

Overcoming of inhibitory effect of aminotriazole by adenine or hypoxanthine. The observation of Hilton that hypoxanthine was less effective than adenine in overcoming the inhibitory effect of 20 mM-aminotriazole in the presence of histidine suggested that the inhibitor could affect one of the enzymes involved in the conversion of hypoxanthine into AMP (Hilton, 1968). Four enzymes should be taken into consideration: a permease, IMP pyrophosphorylase, adenylosuccinate synthetase and adenylosuccinate lyase. It was expected that if a permease or IMP pyrophosphorylase was the point of the inhibitory effect, inosine and adenosine should be equally effective. This experiment is shown in Table 1. However, like with the free bases, the 6-amino derivative appeared to be more effective. To rule out the possibility that hypoxanthine is just a poor purine nucleotide precursor, growth of purine-requiring mutant *purC7* was studied in media containing hypoxanthine or adenine in the presence or absence of aminotriazole. As it can be seen in Table 1, the two purines were equally effective in supporting the growth of the mutant in the absence but not in the presence of aminotriazole.

Lack of aminotriazole effect on the activity of enzyme converting IMP to AMP. The experiment presented in Table 1 did not provide any evidence of aminotriazole interference with purine permeation nor IMP pyrophosphorylase activity. The activity of the two other enzymes involved in the conversion of hypoxanthine to AMP were directly assayed in cell-free extracts in the presence or absence of aminotriazole. The results presented in Table 2 show that aminotriazole had no inhibitory effect on either of the two enzymes.

Table 1

Effects of adenine, adenosine, hypoxanthine and inosine on the growth inhibition by aminotriazole

The growth media were supplemented with 0.2 mM-purine derivatives, 0.1 mM-L-histidine and 10 mM--aminotriazole where indicated, and inoculated at zero time with 10 µg. dry wt. bacteria per ml. After 7 hr. in a shaker at 37° when the growth was still in the logarithmic phase the cell mass was measured turbidimetrically.

Supplements to the medium			Cells grown (µg. dry wt./ml.)	
Purine	Histidine	Aminotriazole	*purC7*	LT-2
Adenine	—	—	371	
Adenine	—	+	151	
Adenine	+	+	257	302
Hypoxanthine	—	—	351	
Hypoxanthine	—	+	71	
Hypoxanthine	+	+	97	97
Adenosine	+	+		186
Inosine	+	+		97

Table 2

Activity of enzymes converting IMP to AMP in the absence and presence of aminotriazole

The activities were assayed in cell-free extracts of mutants *purA1* and *purB12*.

Incubation mixture	Activity (units/mg. protein)	
	Adenylosuccinate synthetase	Adenylosuccinate lyase
No addition	35.0	8.7
Aminotriazole, 10 mM	36.0	8.0

Inhibition of the accumulation of AIR by aminotriazole. The effect of aminotriazole on the earlier steps of purine biosynthesis was first studied by determining the amount of AIR accumulated by non-growing cells of mutant *purC7*. The data in Table 3 show that 10 mM-aminotriazole inhibited the biosynthesis of AIR by about 40%. Further increase in the concentration of aminotriazole did not change appreciably the extent of inhibition. The arylamine assayed was a purine biosynthesis intermediate since adenine added to the incubation medium prevented its formation almost completely. In another experiment it was found that histidine did not decrease the amount of arylamine accumulated. This result indicated that histidine biosynthesis intermediates were not accumulated under the standard experimental conditions. Practically the same results were obtained with either of the two methods of assay, i.e. arylamine or ribosides determination.

Table 3

Arylamine compounds accumulated by the mutant purC7 *in culture medium and cells*

The incubation conditions are described in Methods. The cells collected by centrifugation were disrupted in the laboratory press, as described in Methods for enzyme assays and deproteinized with trichloroacetic acid. The supernatants obtained by centrifugation were used for the arylamine determination.

Incubation mixture	Arylamine compounds in	
	supernatant (mμmoles/ml.)	cells (mμmoles/mg. protein)
Control	92.0	7.5
Aminotriazole, 10 mM	47.8	8.2
Adenine, 0.2 mM	0.3	0.0

The other conclusions from the experiment shown in Table 3 are that the incubated cells contained only a small fraction, about 5%, of the total arylamine and that aminotriazole did not act by blocking the excretion of AIR by the cells to the medium. Therefore, all subsequent determinations of purine intermediates were done in media freed of cells.

Table 4

The effect of aminotriazole on the accumulation of ribose containing compounds by mutants of S. typhimurium

The incubation time was 2 hr., other conditions were the same as in Table 3. Ribose containing compounds were determined in the supernatants.

Mutant	Addition to the incubation mixture	Ribose (mμmoles/ml.)	Inhibition (%)
PurF145	None	12.6	
	Aminotriazole, 10 mM	11.8	0
	Adenine, 0.2 mM	12.0	0
PurD11	None	78	
	Aminotriazole, 10 mM	75	3
	Adenine, 0.2 mM	75	3
PurG310	None	140	
	Aminotriazole, 10 mM	132	5
	Adenine, 0.2 mM	48	66
PurI305	None	75	
	Aminotriazole, 10 mM	77	0
	Adenine, 0.2 mM	14	81
PurC7	None	56	
	Aminotriazole, 10 mM	40	24
	Adenine, 0.2 mM	16	71

Lack of aminotriazole effect on the accumulation of purine biosynthesis inter-mediates earlier than AIR. The observed decrease of AIR accumulation in the in-cubation mixtures in the presence of aminotriazole indicated that an enzyme of purine biosynthesis acting on an intermediafe earlier than AIR was sensitive to the inhi-bitor. In further experiments mutants blocked prior to AIR were used. Since there are no specific methods to determine the intermediates they accumulate, only total ribose compounds in incubation mixtures were determined.

Table 4 presents the results of this experiment. With each mutant two incubations, with and without aminotriazole, were run. In addition, ribose compounds were determined in an incubation mixture containing adenine. The values obtained in these samples served as an approximate correction for ribose compounds unrelated to purine synthesis. As expected, there was no net accumulation of purine interme-diates by mutants *purF* and *purD*, blocked in first and second reactions of purine pathway (5-phosphoribosylamine which could be accumulated by the latter mutant is very unstable even under physiological conditions; Goldwaith, 1956). Only mu-tants *purG*, *purI* and *purC* accumulated significant amounts of ribose compounds which could be attributed to the purine biosynthesis; aminotriazole failed to inhibit the accumulations by cells *purG* and *purI*. Thus the accumulation by the mutant *purC* appeared to be the earliest in the pathway to be affected by aminotriazole. This could mean that the formation of AIR from its immediate precursor, FGAM, would be inhibited by aminotriazole.

DISCUSSION

These experiments were undertaken in order to find an indication as to the aminotriazole-sensitive step of purine biosynthesis in *S. typhimurium*. The results obtained ruled out the enzymes converting IMP to AMP as target of the inhibitory action, suggested by physiological experiments (Hilton, 1968). Instead, it was found that the formation of AIR, but not FGAM, the intermediate just preceding AIR, nor any of the earlier intermediates of the purine pathway, was inhibited. The inhibitory concentrations seem to be rather high: 10 mM-aminotriazole inhibited AIR formation by only 40%. However, it was expected that such high concentration would be needed to produce the inhibition on the basis of physiological experiments (Hilton *et al.*, 1965).

Our results suggest that aminotriazole inhibits the reaction of FGAM cyclization yielding AIR. The reaction is known to require ATP as the only cofactor (Ma-gasanik, 1962). ATP is also required as a cofactor for the earlier steps of purine biosynthesis. Therefore, rather a direct effect on phosphoribosylaminoimidazole synthetase should be taken into consideration.

REFERENCES

Buchanan J. M. (1956). *J. Biol. Chem.* **221**, 1057.

Carter C. & Cohen L. H. (1956). *J. Biol. Chem.* **222**, 17.

Eaton N. R. (1962). *J. Bacteriol.* **83**, 1359.

Goldwaith D. A. (1956). *J. Biol. Chem.* **222**, 1051.

Hilton J. L. (1968). *Weed Science* **16**, 152.

Hilton J. L., Kearney P. C. & Ames B. N. (1965). *Arch. Biochem. Biophys.* **112**, 544.

Kłopotowski T. & Bagdasarian G. (1966). *Acta Biochim. Polon.* **13**, 153.

Kłopotowski T. & Hulanicka D. (1963). *Acta Biochim. Polon.* **10**, 209.

Kłopotowski T. & Wiater A. (1965). *Arch. Biochem. Biophys.* **112**, 562.

Layne E. (1957). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 447. Academic Press Inc., New York.

Lieberman J. (1963). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 6, p. 100. Academic Press Inc., New York.

Lukens L. & Flaks J. (1963). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 6, p. 671. Academic Press Inc., New York.

Magasanik B. (1962). In *The Bacteria* (I. C. Gunsalus & R. Y. Stanier, eds.) vol. 3, p. 295. Academic Press, New York & London.

Vogel H. J. & Bonner D. M. (1956). *J. Biol. Chem.* **218**, 97.

Wiater A. & Krajewska K. (1968). *V FEBS Meeting, Praha, Abstr. Commun.*, p. 89.

HAMOWANIE BIOSYNTEZY AMINOIMIDAZOLORYBOTYDU U *SALMONELLA TYPHIMURIUM* PRZEZ AMINOTRIAZOL

Streszczenie

Doświadczenia nad bezpurynowymi mutantami *Salmonella typhimurium* wskazują na to, że aminotriazol hamuje reakcję cyklizacji rybotydu formyloglicynoamidyny do rybotydu amino-imidazolu.

ALICJA K. DRABIKOWSKA

SUBCELLULAR DISTRIBUTION AND FUNCTION OF UBIQUINONE IN *SALMONELLA TYPHIMURIUM*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, Warszawa 12

1. *Salmonella typhimurium* contains an ubiquinone which on the basis of chromatography with Q_{10} and Q_6 was tentatively identified as Q_8. 2. The ubiquinone is located mainly in particulate fractions, only trace amounts being present in the $105\,000\,g$ supernatant. 3. In the presence of cyanide, endogenous ubiquinone is reduced by NADH or succinate.

Bacteria appear to be particularly suitable organisms for the study of components of electron transport and their participation in respiratory processes since the enzymic composition of these cells can be modified to a large extent by conditions of cultivation. Although the respiratory chain enzymes of bacteria are only similar to, but not identical with, those of mammalian mitochondria, the electron transport in several bacteria and in animals seems to have a common mechanism.

Evidence for participation of quinones as electron carriers in the respiratory chain of various organisms has been given by Green & Brierley (1965) and Brodie (1965); however, their role and significance in electron transport chain so far has not been elucidated (Chance, 1965; Redfearn, 1966; Kröger & Klingenberg, 1966). In the present paper the localization and function of ubiquinone in *Salmonella typhimurium* was studied.

MATERIALS AND METHODS

Growth of bacteria. Salmonella typhimurium wild type strain LT-2 was grown with aeration at 37° in the medium E of Vogel & Bonner (1956) to which were added (per 1 litre): $CoSO_4 \cdot 6H_2O$, 130 µg.; $FeCl_3$, 480 µg.; $CuCl_2 \cdot H_2O$, 270 µg.; $ZnCl_2$, 2000 µg.; H_3BO_3, 290 µg.; and $(NH_4)_2MoO_4 \cdot 4H_2O$, 750 µg. Bacteria were harvested by centrifugation at the beginning of the stationary phase.

Preparation of subcellular fractions. The bacterial cells were washed once with 0.25 M-sucrose - 50 mM-tris-Cl buffer, pH 7.0 (sucrose medium) and suspended in the same medium so as to obtain a protein concentration of 30 mg. per ml. Portions of the cell suspensions, 30 ml., were sonicated in MSE-20 Kc sonicator at maximum

output for 15 min. The mixture was then centrifuged at 1500 *g* to remove undisrupted cells, and the turbid supernatant was centrifuged for 30 min. at 18 000 *g*. The sediment — particles I — was resuspended in sucrose medium, and the remaining turbid supernatant was centrifuged at 105 000 *g* for 3 hr. The obtained pellet — particles II — was resuspended in sucrose medium and the remaining supernatant was called the high-speed supernatant.

Determination of quinone. The procedure of extraction according to Cox & Gibson (1966) was used. The extract was chromatographed on Silica-gel G plates (0.5 mm. thick) with light petroleum (b.p. 40 - 60°) - chloroform (1:4, v/v) as solvent. The area of the much more intensive yellow band was scraped off and eluted with ethyl ether three times. The extracts were collected, concentrated by evaporation and rechromatographed in the above conditions. The well visible yellow band was scraped off, extracted with ethyl ether and the solution evaporated to dryness. The residue was dissolved in anhydrous ethanol and the absorption spectrum was determined in recording Unicam Sp-800 spectrophotometer at wavelengths between 225 and 325 mμ.

The total ubiquinone content and the degree of its reduction were estimated by the method of Pumphrey & Redfearn (1960) with the modification described by Kröger & Klingenberg (1966).

Measurement of respiration. Oxygen uptake was followed polarographically with an oxygen electrode at room temperature after addition of NADH or succinate to suspensions of wall-membrane fragments in 0.25 M-sucrose - 50 mM-tris-Cl buffer, pH 7.0.

Determination of protein. Protein was estimated by the biuret method as described by Szarkowska & Klingenberg (1963).

Chemicals. NADH and Q_{10} were from Sigma Chem. Comp. (St. Louis, Mo., U.S.A.); Q_6 was from Farmochimica Cutolo-Calosi S.p.a. (Napoli, Italy); silica-gel G according to Stahl was from Merck A.G. (Darmstadt, German Federal Republic). Other chemicals used were of analytical grade.

RESULTS

Quinone of Salmonella typhimurium. The extract of quinones chromatographed on silica-gel G plates shows two yellow bands, one with the R_F value of 0.57, and another, less marked band, with the R_F of 0.8 (Fig. 1). After purification by rechromatography the substance from the first band shows a spectrum characteristic of ubiquinone with maximum at 275 mμ (Fig. 3). Its difference spectrum (oxidized *minus* reduced with KBH_4) is shown in Fig. 3b.

The isolated ubiquinone (Q_S) chromatographed on silica-gel plates impregnated with a solution of 5% paraffin in light petroleum and developed in an acetone - - water (95:5, v/v) solvent shows an R_F value of 0.74, whereas R_F values of standards of Q_{10} and Q_6 were 0.58 and 0.86, respectively (Fig. 2a). When the chromatograms
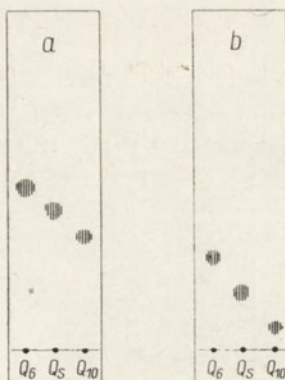
Fig. 1.　　　　　　　　　　　　　　　　Fig. 2.

Fig. 1. Thin-layer chromatography on silica-gel G of quinone separated from lipid extract of *Salmonella typhimurium*. The solvent was light petroleum - chloroform (1:4, v/v).

Fig. 2. Thin-layer chromatography of ubiquinones: *a*, developed with acetone - water (95:5, v/v) solvent; *b*, developed with 95% ethanol. Plates were sprayed with ethanolic solution of reduced methylene blue. Q_6 and Q_{10}, standard samples; Q_s, ubiquinone isolated from *Salmonella typhimurium* cells.
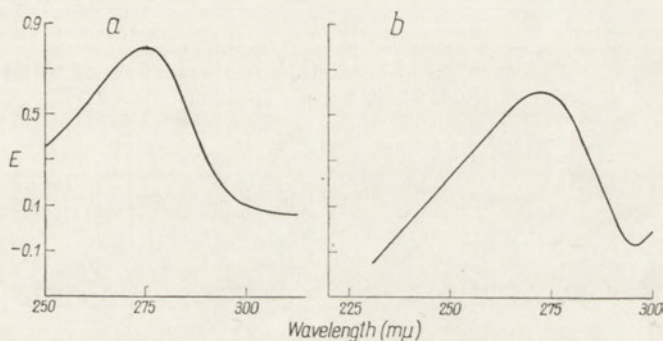

Fig. 3. Spectrum of ethanolic solution of the quinone isolated from *Salmonella typhimurium* cells: *a*, the native form; *b*, difference spectrum (oxidized *minus* reduced by KBH_4).

were developed in 95% ethanol, the R_F values were 0.11, 0.3 and 0.5 for Q_{10}, Q_S and Q_6, respectively (Fig. 2b).

Distribution of ubiquinone. Table 1 shows the localization of ubiquinone in subcellular fractions of *S. typhimurium*. Ubiquinone is bound to the particulate fraction, i.e. to the wall-membrane fragments. The content expressed in mμmoles of ubiquinone per mg. of protein in these fragments, designated as particles I and II, is about twice as high as that in intact cells, while the high-speed supernatant contains only a trace amount. Both kinds of particles obtained by differential centrifugation contain the same amount of ubiquinone. No change of the oxidation-reduction state of the extracted ubiquinone was observed after addition of $FeCl_3$, which proved that the ubiquinone present in cells was in the oxidized form.

Table 1

Distribution of ubiquinone in Salmonella typhimurium cells

Bacterial cells were sonicated, and the cell fractions isolated by differential centrifugation were suspended in 0.25 M-sucrose - 50 mM-tris-HCl buffer, pH 7.0. The extraction of ubiquinone was carried out as described in Materials and Methods.

Preparation	Oxidized ubiquinone (mμmoles/mg. of protein)
Whole cells	0.76
Homogenate (sonicated cells)	0.78
Particles I	1.52
Particles II	1.53
High-speed supernatant (105 000 g)	0.07

Table 2

Oxidation of NADH and succinate by particulate fractions of Salmonella typhimurium cells

Conditions as described in Materials and Methods. The concentration of NADH and succinate was 0.6 and 6 mM, respectively.

Preparation	Substrate	Consumption of O_2 (mμmoles/mg. protein/min.)	Activity ratio: NADH/succinate
Particles I	NADH	200	22.2
	Succinate	9	
Particles II	NADH	230	19.1
	Succinate	12	

Oxidation-reduction changes of ubiquinone. Both kinds of cell particles oxidize NADH and succinate (Table 2). The oxidation rate of NADH was rapid while that of succinate was much slower. Nevertheless, both substrates when added to the suspension of particles in the presence of cyanide caused the reduction of endogenous ubiquinone by about 40 - 50% (Table 3). On the addition of KCN alone there occurred a very pronounced reduction of ubiquinone in preparations of particles I. In this new steady state about a half of ubiquinone was present in the reduced form, and only the remaining half was accessible to reduction by the added substrates. Thus the overall reduction of ubiquinone in particles I amounted to 72% of the amount of the oxidized form present before the addition of cyanide. The reduction of ubiquinone by endogenous substrates in particles II was much less pronounced.

Table 3

Reduction of ubiquinone in particulate fractions of Salmonella typhimurium *cells*

Particles I (19 mg. of protein) or particles II (29 mg. of protein) were suspended in 0.25 M-sucrose - - 50 mM-tris-Cl buffer, pH 7.0; where indicated 5 mM-KCN, 16 mM-succinate or 4 mM-NADH were added; total volume 2.5 ml. The reaction mixture without substrates was kept for 5 min. in a bath at 37°, then the reaction was initiated by the addition of substrates. After 10 min. the reaction was stopped by addition of the mixture of methanol and light petroleum after Kröger & Klingenberg (1966) and ubiquinone estimated according to Pumphrey & Redfearn (1960).

Preparation	Addition	Substrate added	Content of oxidized form of ubiquinone (mμmoles/mg. of protein)	Percentage of reduction
Particles I	None	None	1.80	—
	KCN	None	0.97	—
	KCN	Succinate	0.51	47.5
	KCN	NADH	0.50	48.5
Particles II	None	None	1.85	—
	KCN	None	1.62	—
	KCN	Succinate	0.93	42.5
	KCN	NADH	0.90	44.5

DISCUSSION

Cultures of *Salmonella typhimurium* grown aerobically and harvested in the stationary phase of growth contain one dominant, if not only one, quinone variety. The ethanolic solution of this quinone showed in the ultraviolet a spectrum characteristic of ubiquinone. The obtained spectrum agreed with that reported by Ames (1968) for ubiquinone isolated from *Salmonella* lipids.

The occurrence of ubiquinone in these bacteria is in agreement with observations of Bishop, Pandya & King (1962) who found that the Gram-negative micro-organisms contain ubiquinone but not vitamin K_2.

The comparison of R_F values of the isolated quinone and of standard samples of Q_{10} and Q_6 in our experiments allows to suppose that it comprises eight prenyl units. Such a homologue usually occurs in microbial species (Crane, 1965).

The determination of ubiquinone content in wall-membrane fragments and in the 105 000 *g* supernatant revealed that ubiquinone is located mainly in particulate fractions which contain also other components of the respiratory chain (A. K. Drabikowska, unpublished data). The association of ubiquinone with oxidative enzymes suggests a role for ubiquinone in the electron transport system of the cells. Its amount in whole cells, 0.7 - 0.8 mμmoles per mg. of protein, agrees with that generally found in aerobic bacteria (Crane, 1965). The same amount of ubiquinone in both kinds of particles seems to argue that there is no difference in enzymic composition of these systems to which ubiquinone is bound.

Further evidence for the role of ubiquinone in the succinate and NADH oxidation pathways was provided by studying its oxidation-reduction reactions. The reduction occurs after addition of substrates in the presence of cyanide, but the extent of reduction amounts to 40 - 50% only. Although this indicates that ubiquinone is involved in oxidation processes, there is at present no explanation for the reduction being relatively small.

REFERENCES

Ames G. F. (1968). *J. Bact.* **95**, 833.

Bishop D. H. L., Pandya K. P. & King H. K. (1962). *Biochem. J.* **83**, 606.

Brodie A. F. (1965). In *Biochemistry of Quinones* (R. A. Morton, ed.) p. 355. Academic Press, New York.

Chance B. (1965). In *Biochemistry of Quinones* (R. A. Morton, ed.) p. 459. Academic Press, New York.

Cox G. B. & Gibson F. (1966). *Biochem. J.* **100**, 1.

Crane F. L. (1965). In *Biochemistry of Quinones* (R. A. Morton, ed.) p. 183. Academic Press, New York.

Green D. E. & Brierley G. P. (1965). In *Biochemistry of Quinones* (R. A. Morton, ed.) p. 405. Academic Press, New York.

Kröger A. & Klingenberg M. (1966). *Biochem. Z.* **344**, 317.

Pumphrey A. M. & Redfearn E. R. (1960). *Biochem. J.* **76**, 61.

Redfearn E. R. (1966). In *Vitamins and Hormones* (R. S. Harris, I. G. Wool & J. A. Loraine, eds.) **24**, 465. Academic Press, New York.

Szarkowska L. & Klingenberg M. (1963). *Biochem. Z.* **333**, 674.

Vogel H. J. & Bonner D. M. (1956). *J. Biol. Chem.* **218**, 97.

ROZMIESZCZENIE I ROLA UBICHINONU W KOMÓRKACH
SALMONELLA TYPHIMURIUM

Streszczenie

1. W komórkach *Salmonella typhimurium* występuje ubichinon. Wyniki cienkowarstwowej chromatografii pozwalają przypuszczać, że zawiera on osiem jednostek prenylowych.

2. Ubichinon *S. typhimurium* występuje głównie we fragmentach błon komórkowych; 105 000 *g* supernatant zawiera zaledwie śladowe ilości.

3. Endogenny ubichinon w obecności cyjanku ulega redukcji po dodaniu bursztynianu lub NADH.

BARBARA KOWALSKA

QUANTITATIVE CHROMATOGRAPHY OF AROMATIC AMINO ACIDS ON SEPHADEX G-10

Serum and Vaccine Research Laboratory, ul. Chelmska 30/34, Warszawa 36

1. Phenylalanine, tyrosine and tryptophan were separated from each other and from other amino acids by column chromatography on Sephadex G-10 in 0.5 M-sodium chloride, pH 7.0. 2. Small peptides containing aromatic amino acids were well separated from free amino acids. 3. The recovery of aromatic amino acids from synthetic mixtures, and from acid as well as from enzymic hydrolysates of protein was $100.5 \pm \pm 1.7\%$.

The usefulness of Sephadex gels for separation of substances according to their molecular weights depends to a large extent on the low sorptive properties of cross-linked dextrans. Nevertheless, some deviations from purely molecular sieve effects have been noted already in the early works by Porath (1960) and by Gelotte (1960). It appears that the Sephadex matrix has a marked affinity for aromatic compounds, and this property has been made use of in various chromatographic procedures (Janson, 1967). A successful separation of complex mixtures of phenols has been reported by Woof & Pierce (1967), and of purines, pyrimidines and their compounds by Braun (1967a,b), Zadražil, Šormova & Šorm (1961), Sweetman & Nyhan (1968), and others. Aromatic amino acids are strongly adsorbed on Sephadex, and their presence in the peptides brings about a marked retardation of the peptides on Sephadex columns. This phenomenon has been utilized by Mach & Tatum (1964) for separation of tyrocidines A, B, and C, which differ in the content of tryptophan. Bretthauer & Golichowski (1968) reported that the short, homologous polyphenyl-alanyl peptides were eluted from Sephadex in a reverse order with respect to their molecular weights, showing that the adsorption effect can prevail against the molecular sieve effect.

The adsorption of free aromatic amino acids on Sephadex has been less extensively studied than the behaviour of the peptides containing the aromatic amino acids. Several authors reported a satisfactory separation of iodinated tyrosines (Lissitzky & Bismuth, 1963; Mougey & Mason, 1963). Recently, Eaker & Porath (1967) presented detailed data on the partition coefficients of amino acids, chromatographed on Sephadex G-10 gel using a variety of solvents.

A very large difference in the elution volumes between aromatic and other amino acids suggested a possibility of using Sephadex gels for various preparative and analytical procedures, especially in the domain of protein hydrolysates. Present studies have been undertaken in order to investigate quantitatively the elution patterns of aromatic amino acids present in the synthetic mixtures and in various protein hydrolysates.

MATERIALS AND METHODS

Sephadex G-10 was a Pharmacia (Uppsala, Sweden) product. For the experiments, Sephadex was prepared according to manufacturers' instructions.

Amino acids. Phenylalanine was either a Nutr. Biochem. Corp. (Cleveland, Ohio, U.S.A.) product, or purchased from POCH (Gliwice, Poland). Tryptophan and other amino acids were reagent grade (purchased from POCH) and were used without further purification. Tyrosine was obtained in this laboratory from acid hydrolysate of casein; it was twice crystallized before use. The purity of all amino acids was checked by ascending paper chromatography in *n*-butanol - acetic acid - - water (68:5:27, by vol.).

Protein hydrolysates. Tryptic hydrolysates of casein were a product of Biomed, Serum and Vaccine Manufacturers (Warszawa, Poland). The ratio of amino nitrogen to total nitrogen in these hydrolysates was approximately 50%. Acid hydrolysates of casein were prepared by heating the protein with 6 N-hydrochloric acid in sealed ampoules at 105° for 48 hr.

Spectrophotometry. Extinction was measured on a Unicam SP-500 instrument. Unicam SP-700 was used for recording the spectra. The following molar extinction coefficients were used for calculations: phenylalanine $\varepsilon_{263\ m\mu} = 152$ (Beaven & Holiday, 1952); tyrosine $\varepsilon_{274\ m\mu} = 1400$ (Wetlaufer, Edsall & Hollingworth, 1958); tryptophan $\varepsilon_{280\ m\mu} = 5500$ (Beaven & Holiday, 1952).

Determination of free amino acids. The ninhydrin method as described by Yemm & Cocking (1955) was used.

Chromatography. The technique elaborated by S. Kropiwiec (1966, unpublished) was followed. Sephadex G-10 equilibrated with 0.5 M-sodium chloride, pH 7.0, was poured into 72 cm. ×2.5 cm. glass column with constant stirring. The height of Sephadex bed was always 65 cm. The sample of 6 ml. was placed on top of the bed and pushed into Sephadex with 10 ml. of 0.5 M-sodium chloride, pH 7.0. Elution was started immediately with the same solvent. The elution rate was 0.5 ml./min. Fractions, 4.5±0.1 ml., were collected using either LKB (Stockholm, Sweden) drop counter type 3403B operating in conjunction with LKB fraction collector type 3401B, or the Unipan (Warsaw, Poland) drop counter type 206B with fraction collector type 301. Chromatography was carried out at ambient temperature.

The void volume of the column was determined with Blue Dextran 2000, introduced into the column in a volume of 6 ml. in 0.5 M-sodium chloride, pH 7.0. The effluent was monitored at 263, 274, and 280 mμ. The elution volume of an amino

acid was defined as V_e/V_0, in which V_e denotes the volume of the effluent at which the maximum extinction of a given peak emerged, and V_0 denotes the volume at which the maximum extinction of Blue Dextran 2000 emerged.

RESULTS AND DISCUSSION

Elution of aromatic amino acids

In order to investigate the migration of aromatic amino acids on Sephadex G-10 column, phenylalanine, tyrosine, or tryptophan were dissolved in 6 ml. of 0.5 M--sodium chloride, pH 7.0, and placed on the top of Sephadex G-10. The elution curve was traced at 263, 274, and 280 mμ. At 263 mμ the position of all three amino acids could easily be determined. At 274 and 280 mμ the positions of tyrosine and tryptophan were well apparent but not that of phenylalanine owing to its low extinction coefficient.

Amino acids emerged from the column as normal distribution curves, the median line of which was easily determined for the calculation of the elution volume V_e.

The reproducibility of the elution volume and of the recovery has been studied over a wide range of amino acid concentrations. The results are summarized in Table 1. The recoveries were satisfactory, and largely independent of the amount loaded onto the column. Also, the elution volumes were independent of the amount chromatographed. V_e/V_0 varied by about 5% despite the 70-fold variation in phenylalanine concentration, 10-fold in tyrosine concentration, and 500-fold in tryptophan concentration. A wider range of tyrosine concentrations could not be studied because of its low solubility, and that of phenylalanine because of its low extinction coefficient. A 5% variation in V_e/V_0 is within the limits of variation of the volume of collected fractions.

Partition of the mixture of aromatic amino acids

As shown above, individual aromatic amino acids could be reproducibly chromatographed on Sephadex G-10. In order to investigate the chromatographic behaviour of these amino acids when in mixture, the solutions were prepared containing phenylalanine, tyrosine, and tryptophan in various molal ratios; the total concentration of amino acids in such solutions was between 0.03 and 0.44 mM. A typical elution pattern of a mixture of Phe:Tyr:Trp (3:1:1) is shown in Fig. 1. It is evident that the elution volumes of the aromatic amino acids chromatographed in mixture were the same as when each was run through the column separately (cf. Table 1). Phenylalanine emerged close to tyrosine but the separation was clear even when the elution curve has been traced at 263 mμ, i.e. close to the spectral maximum for phenylalanine but not far from the minimum for tyrosine. However, at the unfavourable molal ratios of the two amino acids, the elution curve had to be traced at two wavelengths, 263 mμ for phenylalanine and 274 or 280 mμ for tyrosine (and

Table 1

Chromatographic parameters of aromatic amino acids on Sephadex G-10 columns

Sephadex G-10 bed (65×2.5 cm.) was equilibrated and eluted at a rate of 0.5 ml./min. with 0.5 M-
-sodium chloride, pH 7.0. All fractions composing a given peak were pooled, and the recovery was
estimated by measuring the extinction at the appropriate wavelengths (263 mμ for phenylalanine,
274 mμ for tyrosine and 280 mμ for tryptophan).

Amino acid	V_e/V_o	Charge (mg.)	Recovery mg.	Recovery %
Phenylalanine	1.84	126.90	120.50	94.9
	1.99	1.96	1.78	90.8
Tyrosine	2.34	7.37	7.32	99.3
	2.22	0.79	0.77	97.5
Tryptophan	5.53	96.90	93.30	96.3
	5.82	0.22	0.24	109.1

Table 2

Recovery of aromatic amino acids from synthetic mixtures after Sephadex G-10 separation

Conditions as for Table 1.

| Amino acid	Phe:Tyr:Trp molal ratio 3:1:1	8:2:1	11:1:5	220:1:11	Mean $\pm$ S.D.
	Recovery (%)				
Phenylalanine	102.2	102.4	92.6	94.4	97.9 ± 2.4
Tyrosine	108.0	109.1	101.9	109.1	107.0 ± 1.7
Tryptophan	91.0	94.7	91.7	102.7	95.0 ± 2.7

Table 3

The purity of phenylalanine and tyrosine from acid hydrolysate of casein separated by Sephadex G-10 gel filtration

The appropriate fractions (see Fig. 2) were pooled and the content of amino acids determined
by two methods. The extinction of phenylalanine was read at 263 mμ and that of tyrosine at 274 mμ.

Amino acid	Method spectrophotometric (a)	ninhydrin (b)	a:b
	μmoles/ml.		
Phenylalanine	1.32	1.41	0.94
Tyrosine	0.24	0.25	0.96

Fig. 1. Chromatography on Sephadex G-10 of the mixture of phenylalanine, tyrosine, and tryptophan. Sephadex G-10 equilibrated and eluted with 0.5 м-sodium chloride, pH 7.0. Elution rate 0.5 ml./min., fractions 4.5 ml. Phenylalanine 18.9 μmoles, tyrosine 5.82 μmoles, tryptophan 6.0 μmoles.
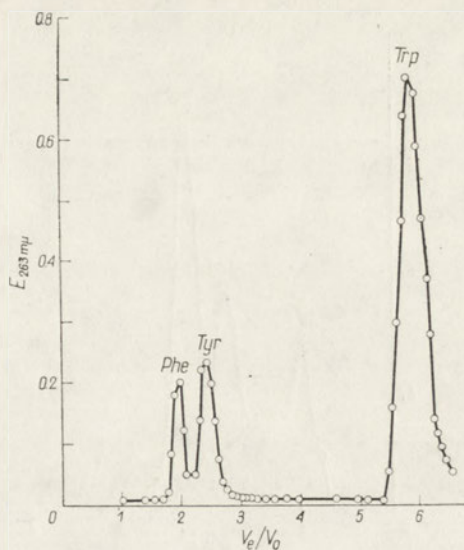
tryptophan). Tryptophan emerged well separated from tyrosine and could be traced at any of the three wavelengths employed.

The recovery of aromatic amino acids, chromatographed in mixture, was quite satisfactory and independent of the molal ratios or the total amount of amino acids placed on the column. As it is shown in Table 2, the recovery was complete with the standard deviation below 3% of the mean. Neither the total amount of amino acids placed on the column, nor their molal ratios affected the recovery.

Separation of aromatic amino acids from protein hydrolysates

A typical elution pattern of acid hydrolysate of casein is shown in Fig. 2. Three peaks are apparent: the first emerging at the void volume of the column; the second at V_e/V_0 1.88, i.e. at the elution volume characteristic of phenylalanine; and the third peak at the elution volume of tyrosine, V_e/V_0 2.26. Tryptophan was absent, due to its destruction during acid hydrolysis. The identity of phenylalanine and of tyrosine in the respective fractions has been confirmed by spectrophotometry. Peak *I* did not exhibit bands characteristic of aromatic amino acids. In order to throw light on the origin of peak *I*, a mixture of crystalline amino acids, simulating the composition of casein (Hannig, 1956), was chromatographed under the same conditions. Peak *I* appeared in the same position (Fig. 3) and its absorption spectrum (Fig. 3, inset) showed no aromatic absorption bands. Similarly, paper chromatography did not reveal the presence of aromatic amino acids. Thus it appears that various, possibly all, non-aromatic amino acids present in the protein hydrolysate emerged in peak *I*. The absorption of this fraction in the ultraviolet presumably results mainly from the presence of cystyl and histydyl chromophores.
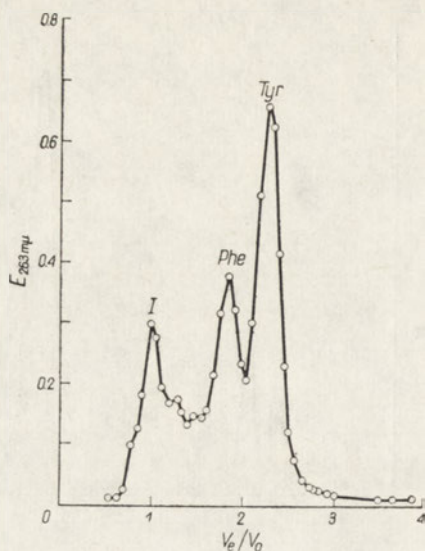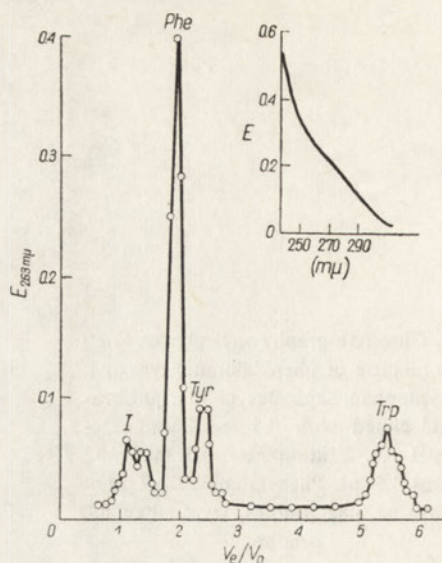
Fig. 2.

Fig. 3.

Fig. 2. Chromatography on Sephadex G-10 of acid hydrolysate of casein. Conditions as under Fig. 1; 120 mg. of protein hydrolysate placed on the column.

Fig. 3. Artificial mixture, simulating acid casein hydrolysate, chromatographed on Sephadex G-10. Conditions as under Fig. 1. The content of phenylalanine was markedly in excess of that in the native casein hydrolysate. The inset shows the ultraviolet absorption spectrum of the unretarded peak *I*.
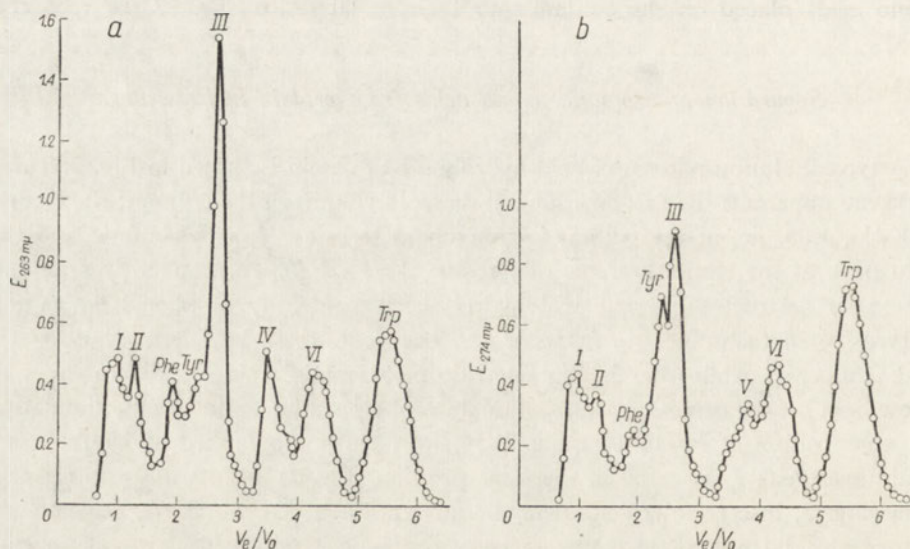

Fig. 4. Chromatography on Sephadex G-10 of tryptic digest of casein. *a*, Effluent monitored at 263 mμ; *b*, effluent monitored at 274 mμ. Conditions as under Fig. 1; 60 mg. of the digest placed on the column.

In order to find out whether non-aromatic amino acids contaminate tyrosine and phenylalanine fractions, the samples composing appropriate peaks were pooled, and the concentration of tyrosine or of phenylalanine was determined by ninhydrin assay and by spectrophotometric measurement. Obviously, in the absence of contamination with foreign substances, the ratio of the concentrations found by the two methods should equal unity. The results presented in Table 3, show that the index of purity of phenylalanine was 0.94 and that of tyrosine 0.96. Paper chromatography confirmed the absence of non-aromatic amino acids in both fractions analysed.

Tryptic hydrolysate of casein presented a more complicated pattern of elution. The plot of extinction at 263 mμ exhibited seven peaks (Fig. 4a). Peak *I* emerged at the void volume of the column, and was closely followed by peak *II*. The next peak emerged at the elution volume characteristic of phenylalanine, and was followed by peak *III*, on the ascending arm of which a small inflection appeared at a position characteristic of tyrosine. Between peak *III* and the last peak to emerge, that of tryptophan, two major peaks *IV* and *VI* are apparent. When the extinction was measured at 274 mμ (Fig. 4b) the distribution of the peaks was similar but the phenylalanine peak was decreased while the tyrosine peak was better separated. Peak *IV*, which was evident at 263 mμ, almost disappeared and was replaced by peak *V* at a somewhat higher elution volume. This peak was also apparent at 280 mμ. Beyond the tryptophan peak, no other ultraviolet absorbing material emerged from the column.

Spectrophotometric analysis of peak *I* failed to reveal the presence of aromatic absorption bands. Neither have these bands been found in most of the fractions comprising peak *II* with the exception of the last segment of its descending arm, beginning with V_e/V_0 1.4, where an inflection indicated the presence of a small amount of aromatic amino acids. These fractions have not been analysed in more detail, but their spectral profiles taken together with their elution volumes suggest that they might contain peptides incorporating aromatic amino acids.

The absorption spectra of peaks *III*, *IV*, and *VI* (see Fig. 4a) are presented in Fig. 5a, b, and c, respectively. Although each exhibits a prominent absorption band in the aromatic region, none is characteristic of the aromatic amino acids. The maximum absorbancies of peaks *III*, *IV*, and *VI* are at 262 mμ, 250 mμ and 268 mμ, respectively. It seems likely that in these peaks the material is accumulated which derives from the degradation products of nucleic acids which have been introduced into the protein hydrolysate with the homogenate of pancreas used for digestion of casein.

In the elution pattern presented in Fig. 4b, a peak is apparent at V_e/V_0 3.8 (peak *V*), which can be detected at 274 mμ (and also at 280 mμ) but not at 263 mμ. Its absorption spectrum, shown in Fig. 6, indicates the presence of tyrosyl, and possibly tryptophyl, residues. It seems likely that peak *V* contained the peptides relatively rich in aromatic amino acids, which retarded the migration of the peptides through the column, similarly to what has been observed in the case of polyphenylalanyl peptides (Bretthauer & Golichowski, 1968).
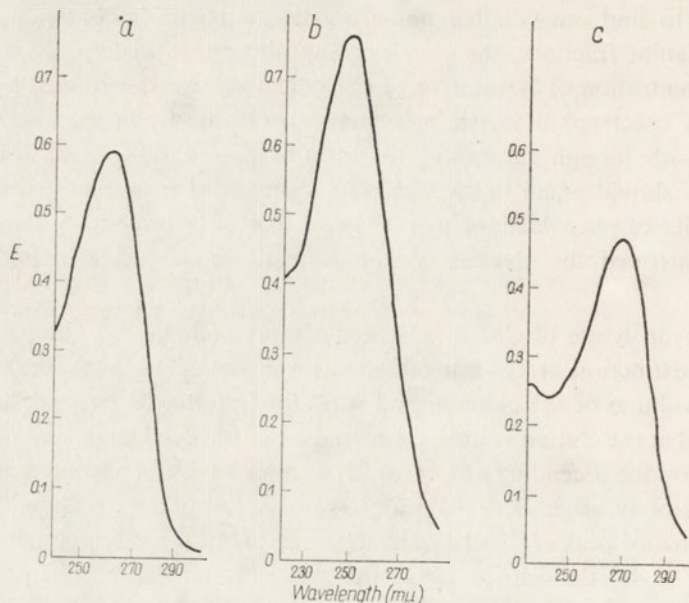
Fig. 5. Ultraviolet absorption spectra of no amino acid containing fractions from tryptic digest of casein (cf. Fig. 4a). *a*, Peak *III*; *b*, peak *IV*; *c*, peak *VI*.
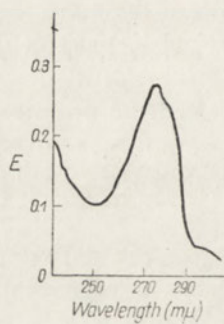

Fig. 6. Ultraviolet absorption spectrum of peak *V* (cf. Fig. 4b).

The purity of the aromatic amino acids separated from enzymic digest of casein has been analysed by means of Sephadex chromatography (Table 4). Tyrosine and tryptophan fractions appear free of contamination; this was also confirmed by paper chromatography. The phenylalanine fraction is less pure, possibly due to the contamination by non-identified ultraviolet absorbing products.

Direct estimation of the recovery of aromatic amino acids present in protein hydrolysates was not feasible, owing to a large amount of other ultraviolet absorbing substances present in the hydrolysates. An indirect method, therefore, has been resorted to. The hydrolysate was first chromatographed, and the amount of free phenylalanine, tyrosine, and tryptophan determined, as described above. The same hydrolysate was then supplemented with known amounts of three aromatic amino

Table 4

The purity of aromatic amino acids from tryptic digest of casein separated by Sephadex G-10 gel filtration

Appropriate fractions (see Fig. 4) were pooled and the content of amino acids determined by two methods. The extinction of phenylalanine was read at 263 mμ, of tyrosine at 274 mμ, and that of tryptophan at 280 mμ.

Amino acid	Method		a:b
	spectro-photometric (a)	ninhydrin (b)	
	μmoles/ml.		
Phenylalanine	0.77	0.61	1.26
Tyrosine	0.13	0.12	1.08
Tryptophan	0.026	0.029	0.90

Table 5

Recovery of aromatic amino acids from acid and enzymic hydrolysates of casein

Hydrolysate	Amino acid	Assayed mixture			Recovery	
		Hydro-lysate (mg.)	Supple-ment (mg.)	Total (mg.)	(mg.)	(%)
Acid	Phenylalanine	5.43	8.20	13.63	13.76	101
	Tyrosine	2.17	0.61	2.78	2.83	102
Tryptic	Phenylalanine	3.15	2.65	5.80	5.98	103
	Tyrosine	0.64	0.49	1.13	1.19	105
	Tryptophan	0.52	0.56	1.08	1.10	93

acids, and chromatographed again. The ratio of the amount of free aromatic amino acids found prior to and after supplementation was considered as index of recovery. The results are summarized in Table 5. It is evident that the recoveries of phenylalanine, tyrosine, and tryptophan are very close to 100%. The mean recovery of the three aromatic amino acids, whether from synthetic mixtures (cf. Table 2) or from acid and tryptic hydrolysates (cf. Table 5) was calculated to be 100.5±1.7 S.D.

CONCLUSIONS

Aromatic amino acids exhibit a typical chromatographic behaviour on Sephadex G-10. Under the conditions employed in the present studies, i.e. at neutral pH and at a slightly elevated ionic strength, phenylalanine, tyrosine, and tryptophan migrate through the column at a remarkably reproducible rate, independent of the amount of accompanying other amino acids and small peptides. The molecular

sieve effect is negligible, if any, during the chromatography of aromatic amino acids on Sephadex G-10.

The propensity of Sephadex gel for the binding of aromatic amino acids together with the satisfactory quantitative parameters of the chromatography based upon this effect, offer interesting possibilities for preparative and analytical work. Protein hydrolysates, such as used e.g. in nutritional studies, can be freed of all or some of the aromatic amino acid in a simple, one-stage procedure. Determination of tryptophan in proteins could be considerably facilitated by partitioning enzymic hydrolysates of proteins on Sephadex column prior to spectrophotometric measurements.

REFERENCES

Beaven G. H. & Holiday E. R. (1952). *Advanc. Protein Chem.* **7**, 324.
Braun R. (1967a). *Biochim. Biophys. Acta* **142**, 267.
Braun R. (1967b). *Biochim. Biophys. Acta* **149**, 601.
Bretthauer R. K. & Golichowski A. M. (1968). *Biochim. Biophys. Acta* **155**, 549.
Eaker D. & Porath J. (1967). *Separat. Sci.* **2**, 507.
Gelotte B. (1960). *J. Chromat.* **3**, 330.
Hannig K. (1956). In *Biochemisches Taschenbuch* (H. M. Rauen, ed.) p. 196. Springer Verlag, Berlin.
Janson J. C. (1967). *J. Chromat.* **28**, 12.
Lissitzky S. & Bismuth J. (1963). *Clin. chim. Acta* **8**, 269.
Mach B. & Tatum E. L. (1964). *Proc. Nat. Acad. Sci. Wash.* **52**, 876.
Mougey E. H. & Mason J. W. (1963). *Analyt. Biochem.* **6**, 223.
Porath J. (1960). *Biochim. Biophys. Acta* **39**, 193.
Sweetman L. & Nyhan W. L. (1968). *J. Chromat.* **32**, 662.
Woof J. B. & Pierce J. S. (1967). *J. Chromat.* **28**, 94.
Wetlaufer D. B., Edsall J. T. & Hollingworth B. R. (1958). *J. Biol. Chem.* **233**, 1421.
Yemm E. W. & Cocking E. C. (1955). *Analyst* **80**, 209.
Zadražil S., Šormova Z. & Šorm F. (1961). *Coll. czech. chem. Commun.* **26**, 2643.

ILOŚCIOWA CHROMATOGRAFIA AMINOKWASÓW AROMATYCZNYCH NA SEPHADEX G-10

Streszczenie

1. Fenyloalaninę, tyrozynę i tryptofan oddzielono od siebie i od innych aminokwasów przy pomocy kolumnowej chromatografii na Sephadex G-10 w 0,5 M-chlorku sodu, pH 7.0.

2. Małe peptydy, zawierające aminokwasy aromatyczne, były dobrze oddzielone od wolnych aminokwasów.

3. Odzyskanie aminokwasów aromatycznych ze sztucznych mieszanin oraz z kwaśnych i enzymatycznych hydrolizatów białka wynosiło 100,5 ± 1,7%.

JOLANTA GRABAREK-BRALCZYK, K. KLECZKOWSKI and I. REIFER

SYNTHESIS OF ARGININE FROM CITRULLINE AND ASPARTATE IN PREPARATIONS FROM WHEAT SPROUTS

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, Warszawa 12

1. Arginine synthesis from citrulline and aspartate in preparations from wheat sprouts was studied. The method used for separation of the two arginine-synthesizing enzymes from animal liver proved to be unsuitable for separation of the plant enzymes. 2. Attempts to separate the two enzymes by other methods were unsuccessful, therefore they were isolated together in one preparation from acetone-dried extract, and purified 16-fold. 3. Some kinetic properties of argininosuccinate synthetase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1) are given. No remarkable differences were found between plant enzymes and enzymes from animals and micro-organisms as reported in literature.

The mechanism of arginine synthesis from citrulline and aspartate in mammals was described many years ago (see review by Ratner, 1954). In higher plants, however, this metabolic path was not studied in detail. Kasting & Delwiche (1955) feeding water-melon seedlings, and Coleman & Hegarty (1957) feeding barley and white clover seedlings with [^{14}C]ornithine found the label in citrulline and arginine. Kleczkowski (1957, 1958) and Reifer & Buraczewska (1958) showed that excised pea sprouts when fed with citrulline metabolized this amino acid to arginine. Buraczewski, Kleczkowski & Reifer (1960) demonstrated that acetone-powder extracts from pea sprouts catalysed reversibly the conversion of argininosuccinate to arginine and fumarate. In further work Fotyma, Kleczkowski & Reifer (1961) demonstrated the synthesis of arginine from citrulline and aspartate in crude extracts from wheat, but not from pea sprouts. It appeared (Grabarek, Reifer & Kleczkowski, 1965) that a natural inhibitor of arginine synthesis was present in homogenate from pea sprouts, and that it could be separated on Sephadex G-25.

In previous work Kleczkowski & Grabarek-Bralczyk (1968) have reported about a new path of arginine synthesis from carbamoylaspartate and ornithine in plant preparations. In this paper we present some results concerning the "classical" path of arginine synthesis from citrulline and aspartate in preparations from wheat sprouts. Some kinetic characteristics of argininosuccinate synthetase [L-citrulline : L-aspartate ligase (AMP), EC 6.3.4.5] and argininosuccinate lyase (L-argininosuccinate arginine-lyase, EC 4.3.2.1) are also reported.

MATERIALS AND METHODS

Reagents. These were from the following sources: L-citrulline and ATP-Na (Reanal, Budapest, Hungary); L-aspartic acid (Fluka, Buchs, Switzerland); ATP-Na, DL-α- and DL-β-methylaspartic acid, and L-argininosuccinate barium salt (Sigma Chem. Comp., St. Louis, Mo., U.S.A.); urease, 250 units/mg. (Merck, Darmstadt, German Federal Republic). All other reagents were of Polish origin, purchased from Biuro Obrotu Odczynnikami Chemicznymi (Gliwice, Poland). Arginase was prepared from bovine liver according to Ratner (1955). L-Argininosuccinate barium salt was converted to potassium salt by adding 1.8 ml. of 0.5 M-potassium sulphate per 20 micromoles. Sephadex G-25 medium was from Pharmacia (Uppsala, Sweden).

Determinations of ASA-synthetase activity. As we were unable to separate ASA-synthetase[1] from ASA-lyase (see Results), the activity of the former had to be determined in the presence of the latter. Therefore argininosuccinate could not be measured, and the arginine formed as the final product was determined. The incubation mixture was as follows: 50 - 100 µl. of crude extract (1 - 2 mg. of protein in homogenate and 120 µg. in acetone-powder extract) or partially purified enzyme preparation containing ASA-synthetase and ASA-lyase (10 - 45 µg. of protein), 6.25 mM-L-citrulline, 6.25 mM-L-aspartate, 6.25 mM-ATP, 12.5 mM-MgSO$_4$, 125 mM-K-phosphate buffer, pH 7.2, and water to a total volume of 0.8 ml. After incubation for 40 min. at 37°, the samples were inactivated with 0.5 ml. of 1 N-HCl, mixed and neutralized with 0.5 ml. of 1 N-NaOH, then 20 units of urease and 20 units of arginase were added, and incubated again for 30 min. at 37°. In the control sample, L-citrulline and aspartate were omitted. The synthesis of argininosuccinate was calculated from the amount of liberated ammonia using Conway vessel technique (Conway, 1947). The enzyme activity was expressed as micromoles of arginine synthesized per mg. of protein under conditions of assay.

Determination of ASA-lyase activity. The incubation mixture contained: 50 - 200 µl. of partially purified enzyme preparation (40 - 60 µg. of protein), 12.5 mM-L-argininosuccinate, 125 mM-K-phosphate buffer, pH 7.2, and water to a total volume of 0.8 ml. The control sample was prepared without the addition of L-argininosuccinate. The time of incubation was 30 min. at 37°. All further procedure was as described under assay of ASA-synthetase. The enzyme activity was expressed in micromoles of arginine formed from ASA per mg. of protein under conditions of assay.

Protein determination. This was done according to Lowry, Rosebrough, Farr & Randall (1951).

---

[1] Abbreviations: ASA, argininosuccinate; ASA-lyase, argininosuccinate lyase; ASA-synthetase, argininosuccinate synthetase; PCMB, *p*-chloromercuribenzoate.

RESULTS

Similarly as it has been observed for pea sprouts (Grabarek *et al.*, 1965), also in the crude homogenates from clover and sunflower sprouts the synthesis of arginine could not be demonstrated unless the natural inhibitor was separated by Sepha-

Table 1

Arginine synthesis from citrulline and aspartate in crude plant homogenates and after Sephadex G-25 gel filtration

Incubation mixture as described under Methods for determination of ASA-synthetase.

Plant sprouts	Age of sprouts (days)	Arginine (μmoles/mg. protein)	
		Homogenate	
		crude	after gel filtration
Green pea	7	0	0.21
Barley	4	0.24	0.35
White clover	10	0	0.16
Yellow lupin (bitter)	7	0.05	0.24
Yellow lupin (bitter)	14	0.03	0.20
Blue lupin	5	0.18	0.31
Cucumber	13	0.02	0.06
Oats	6	0.03	0.09
Wheat v. Dańkowska 40	5	0.42	0.63
Wheat v. Dańkowska 40	10	0.45	0.70
Wheat v. Opolska	5	0.18	0.32
Wheat v. Opolska	10	0.22	0.36
Ryegrass	6	0.06	0.38
Sunflower	16	0	0.15

Table 2

Arginine synthesis in crude homogenates and extracts from acetone-dried preparations from wheat sprouts

Incubation mixture as described under Methods for determination of ASA-synthetase.

Arginine (μmoles/mg. protein)	
Crude homogenate	Acetone-powder extract
0.82	2.25
0.64	1.93
0.80	2.11
0.82	2.30
0.36	1.60
0.47	1.65

dex G-25 gel filtration (Table 1). Wheat sprouts (variety Dańkowska 40) showed the highest activity and therefore this material was used in the present experiments.

The arginine-synthesizing activity was about three times higher in acetone-
-powder extracts than in crude homogenates (Table 2). Acetone-dried preparations from wheat sprouts homogenate kept at -10° retained full activity for two months. After four months of storage, a 50% loss in activity was observed.

ASA-synthetase and ASA-lyase preparation

The method described for separation of ASA-synthetase from ASA-lyase in liver homogenates could be applied also for the acetone-dried rat-liver preparation (Ratner & Pappas, 1949a,b; Ratner & Petrack, 1953a,b); similarly as from the homogenate, ASA-lyase was precipitated at 0 - 0.3 ammonium sulphate saturation and ASA-synthetase at 0.4 - 0.5 saturation. This procedure, however, proved to be ineffective both with the homogenate from wheat sprouts and the acetone-dried preparation, as in either case the two enzymes were found in fractions of 0.5, 0.6 and 0.7 ammonium sulphate saturation.

For partial purification of the two enzymes catalysing the formation of arginine from citrulline and aspartate, the following procedure was used. The acetone-dried powder from 6 - 8-day old wheat sprouts was extracted with 0.1 M-tris maleate -
- NaOH buffer, pH 7.2 (50 ml. per 1 g.), and the supernatant fractionated with solid ammonium sulphate to 0.3, 0.5 and 0.7 saturation. The precipitate between 0.5 and 0.7 saturation was dissolved in 0.2 M-K-phosphate buffer pH 7.2, and again fractionated with ammonium sulphate to 0.3, 0.55 and 0.7 saturation. The precipitate between 0.55 and 0.7 saturation was collected, dissolved in the above phosphate buffer and freed from ammonium sulphate on Sephadex G-25. All steps of the procedure were carried out in a cold room at 4°. The results of purification are shown in Table 3; the purification obtained was 16-fold, and the

Table 3

Partial purification from wheat sprouts of enzymes synthesizing arginine from citrulline and aspartate

Incubation mixture as described under Methods for determination of ASA-synthetase. For details of purification see Text. Ammonium sulphate was removed on Sephadex G-25.

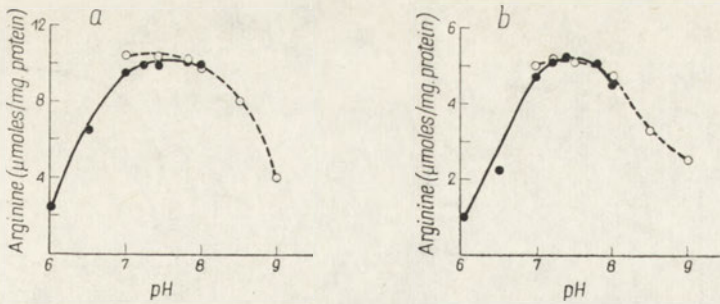| Step of preparation	Volume (ml.)	Total protein (mg.)	Total activity (μmoles arginine)	Specific activity (μmoles arginine/ /mg. protein)	Purifi- cation factor	Yield (%)
Extract of acetone powder	500	600	1122	1.87	1	100
Ppt. at 0.5 - 0.7 ammonium sulphate sat.	100	60	753	9.41	5	67
Ppt. at 0.55 - 0.7 ammonium sulphate sat.	25	7.5	224	29.84	16	20

Fig. 1. Effect of pH on the activity of (*a*), ASA-synthetase, and (*b*), ASA-lyase. Assay condition as described under Methods; (●), 0.2 M-phosphate buffer; (○), 0.2 M-tris-HCl buffer. Protein concentration for ASA-synthetase was 30 μg./sample and for ASA-lyase 40 μg./sample.

yield 20%. In comparison with the activity in crude homogenates, 53-fold purification of both ASA-synthetase and ASA-lyase was achieved.

The preparation, containing 1 - 5 mg. of protein per 1 ml., was used for further experiments. It could be stored in small portions at -15° to -20° for 6 - 8 weeks without loss of activity but a second freezing-thawing cycle resulted in considerable inactivation of the enzymes.

The attempts to separate ASA-synthetase from ASA-lyase in the obtained preparations using DEAE-cellulose and hydroxyapatite chromatography, Sephadex gel filtration as well as adsorption on aluminium gel and further elution, were ineffective. It was possible to separate ASA-lyase from ASA-synthetase, with very low yield, but ASA-synthetase fractions always contained the lyase activity.

The optimum pH value both for ASA-synthetase and ASA-lyase was found to be between 7.0 and 8.0 (Fig. 1a, b). It was therefore possible to study the properties of ASA-synthetase by measuring the formation of arginine.

Some properties of ASA-synthetase

The enzyme activity was linear with the incubation time up to 50 min. (Fig. 2a) and with protein concentration up to at least 55 μg. (Fig. 2b). The effect of tem-

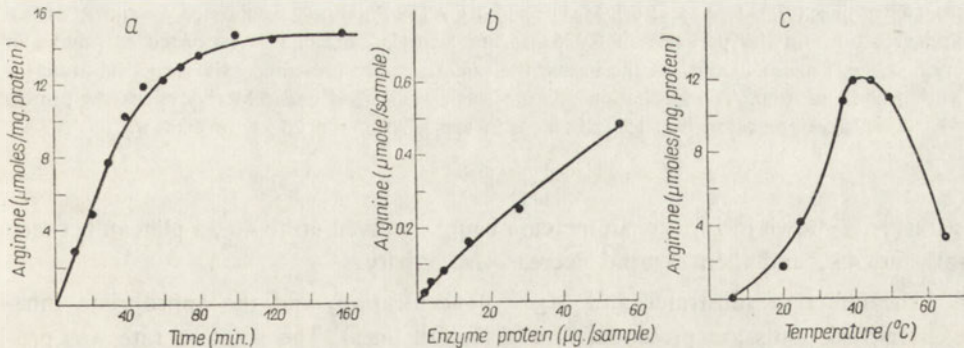Fig. 2. Dependence of ASA-synthetase activity on (*a*), incubation time (25 μg.protein/sample); (*b*), enzyme protein concentration; and (*c*), temperature (40 μg. protein/sample). Other conditions as described under Methods.
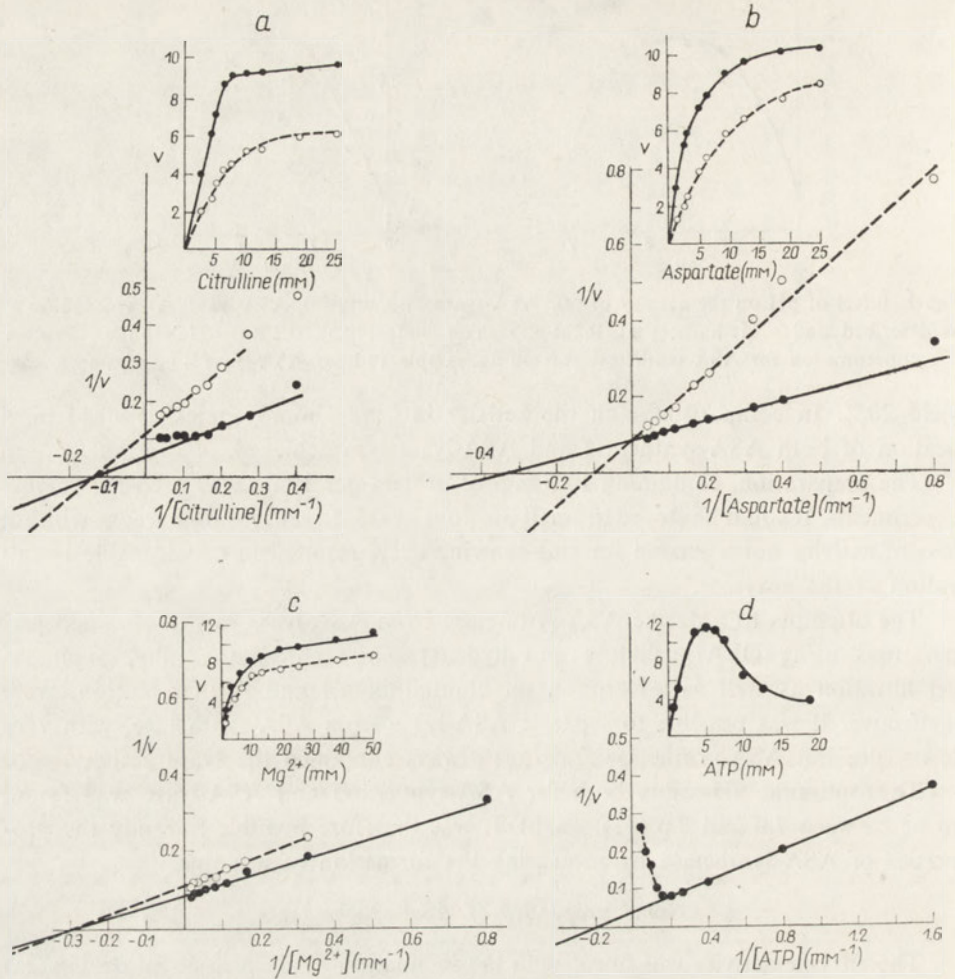
Fig. 3. Lineweaver-Burk plots for ASA-synthetase activity with increasing concentrations of: (*a*), L-citrulline; (*b*), L-aspartate; (*c*), Mg^{2+}; and (*d*), ATP. Full lines, uninhibited reactions; dashed lines, reactions in the presence of 0.026 mM-α-DL-methylaspartate. *v* is expressed as μmoles of arginine/mg. protein/40 min. In the insets, the same data are presented as *v versus* substrate (or Mg^{2+}) concentration. The incubation mixture was as described under Methods; enzyme protein concentration was 15 μg./sample in expts. *a - c*, and 25 μg. in expt. *d*.

perature is shown in Fig. 2c, an increase being observed up to 40°, a plateau between 40° and 45°, and then a rapid decrease in activity.

The effect of substrates and Mg^{2+} concentration, and the appropriate Lineweaver-Burk plots are presented in Fig. 3 (full lines). The reaction rate was proportional with concentration for citrulline, aspartate and Mg^{2+} up to 6.25 mM, and for ATP up to 4 mM with a plateau between 4 and 7 mM. Higher ATP concentrations caused a drop in activity.
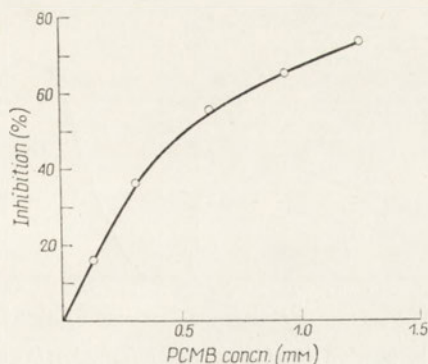
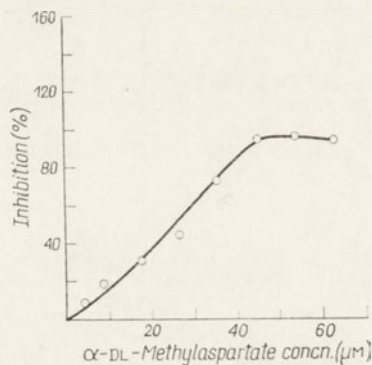Fig. 4.   Fig. 5.

Fig. 4. Effect of PCMB concentration on ASA-synthetase activity. Assay conditions as described under Methods (30 μg. protein/sample).

Fig. 5. Effect of α-DL-methylaspartate concentration on ASA-synthetase activity. Assay conditions as described under Methods (20 μg. protein/sample).

K_m values calculated from the Lineweaver-Burk plots were found to be for citrulline 7.1 mM, for aspartate 2.9 mM, for ATP 5.5 mM, and for Mg^{2+} 3.3 mM.

The following divalent metal ions were tested as possible inhibitors of ASA-synthetase: Co^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+} and Cu^{2+} in concentrations of 6.25 - - 25 mM. The most potent inhibitors were Co^{2+} and Ca^{2+}. At 6.25 and 25 mM concentrations, Co^{2+} inhibited by 34 and 88%, respectively. Ca^{2+} caused inhibition of the same order of magnitude. Potassium cyanide at concentrations of 6.25, 12.5 and 25 mM inhibited, respectively by 39, 49 and 62%. PCMB at 1.25 mM concentration inhibited ASA-synthetase by 70% (Fig. 4). α-DL-Methylaspartate at 0.0525 mM concentration gave full inhibition (Fig. 5) but β-DL-methylaspartate had no effect. To establish the type of inhibition by α-DL-methylaspartate with respect to citrulline, L-aspartate and Mg^{2+}, experiments were carried out with constant inhibitor concentrations and varying concentrations of the above substrates and Mg^{2+}. The graphic interpretation of these experiments using Lineweaver-Burk plots are shown in Figs. 3 a-c (dashed lines). As can be seen, α-DL-methylaspartate is a competitive inhibitor with respect to aspartate, and a non-competitive one with respect to citrulline and Mg^{2+}. K_i with respect to aspartate was found to be 0.0097 mM, to citrulline 0.024 mM, and to Mg^{2+} 0.095 mM.

Some properties of ASA-lyase

The enzyme activity was linear with time up to 20 min. of incubation (Fig. 6a), and with protein concentration up to at least 100 μg. of protein per sample (Fig. 6b). The optimum temperature (Fig. 6c) ranged from 30° to 40°, above 40° a sharp decrease in activity being observed. The effect of substrate concentration is shown in Fig. 7; a linear dependence was observed up to 6.25 mM. The K_m value calculated from Lineweaver-Burk plots was for ASA 9 mM.
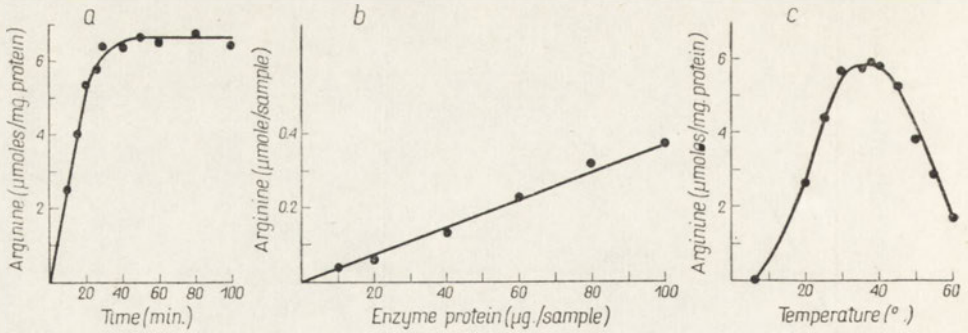
Fig. 6. Dependence of ASA-lyase activity on (*a*), incubation time (40 μg.protein/sample); (*b*), enzyme protein concentration; and (*c*), temperature (40 μg. protein/sample). Other conditions as described under Methods.
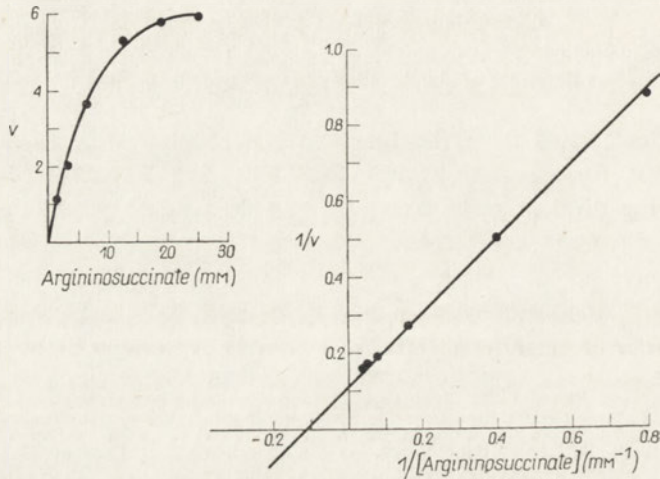

Fig. 7. Lineweaver-Burk plot for ASA-lyase activity with increasing concentration of argininosuccinate. *v* is expressed as μmoles arginine/mg. protein/40 min. In the inset, the same data are presented as *v versus* substrate concentration. Assay conditions as described under Methods (45 μg. protein/sample).

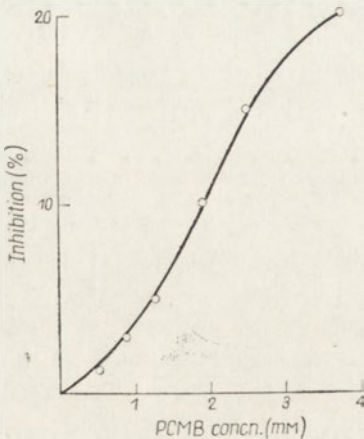Fig. 8. Effect of PCMB concentration on ASA-lyase activity. Assay conditions as described under Methods (60 μg. protein/sample).

Of the metal ions studied, the highest inhibitory effect was shown by Fe^{2+}, 48% at 6.25 mM and 68% at 25 mM concentration. The effectiveness of inhibition by other metal ions with respect to Fe^{2+} decreased in the following order: $Fe^{2+} > > Cu^{2+} > Zn^{2+} > Co^{2+} > Ca^{2+}$. KCN at 25 mM concentration inhibited ASA-lyase by 19%. PCMB (Fig. 8) showed only a slight inhibitory effect (20% at 3.75 mM concentration). β-DL-Methylaspartate slightly inhibited ASA-lyase, whereas α-DL-methylaspartate, in contrast to its action on ASA-synthetase, had no effect.

DISCUSSION

The mechanism of arginine synthesis from citrulline and aspartate first demonstrated on mammals (Ratner, 1954) was confirmed in reptiles (Brown & Cohen, 1959; Brown, Brown & Cohen, 1959) and micro-organisms (De Deken, 1962; Wampler & Fairley, 1967). Our results presented in this paper show that the same mechanism is operative in higher plants, and are comparable with the data presented for liver by Ratner & Pappas (1949a,b), Ratner & Petrack (1951, 1953a,b) and Ratner (1954), and for *Neurospora crassa* by Wampler & Fairley (1967).

The K_m values of ASA-synthetase for L-aspartate, L-citrulline, ATP and Mg^{2+}, as well as those of ASA-lyase, are in agreement with the data reported previously for mammalian enzymes (Table 4). In more highly purified preparations from mammalian livers, these values for L-aspartate and L-citrulline were about 100 times lower (Ratner, 1962).

The optimum pH for both plant enzymes was found to be between 7.0 and 8.0. These results are in agreement with those of Wampler & Fairley (1967) for *Neurospora crassa* ASA-synthetase and differ from those of Ratner & Petrack (1953a) for the separated mammalian enzyme (pH 8.5 - 8.7). However, the mammalian ASA-lyase has the same pH optimum as the plant enzyme.

Table 4

Comparison of K_m *values* (M) *for ASA-synthetase and ASA-lyase from various sources*

K_m for	Liver			*Neurospora crassa*	Wheat
	Ratner & Petrack (1953b)	Ratner (1962)[*]	Rochovansky & Ratner (1967)[*]	Wampler & Fairley (1967)	Present results
L-Citrulline	1.2×10^{-3}	4.1×10^{-5}	4.6×10^{-5}	6.8×10^{-3}	5.4×10^{-3}
L-Aspartate	1.2×10^{-3}	3.7×10^{-5}	3.8×10^{-5}	1.07×10^{-5}	3.8×10^{-3}
Mg^{2+}	1.0×10^{-3}	—	—	—	2.2×10^{-3}
ATP	—	3.2×10^{-4}	—	—	5.5×10^{-3}
ASA	1.5×10^{-3}[**]	—	—	—	9.1×10^{-3}

[*] Highly purified preparation.
[**] After Ratner, Anslow & Petrack (1953).

ASA-synthetase from wheat sprouts was inhibited competitively by 100% by α-DL-methylaspartate at 0.0525 mM concentration, this being in agreement with the results reported by Cimino, Venuta & Salvatore (1966) and Severina (1962) for the mammalian enzyme.

Contrary to Cimino *et al.* (1966), lower concentrations of α-DL-methylaspartate did not stimulate the plant ASA-synthetase. β-DL-Methylaspartate had no effect on plant ASA-synthetase and showed only a slight inhibitory effect on ASA-lyase. As reported recently by Rochovansky & Ratner (1967), β-DL-methylaspartate can act as substrate for ASA-synthetase but the condensation product with citrulline acts as an inhibitor of ASA-lyase.

Plant ASA-synthetase similarly as the mammalian enzyme was inhibited in the same degree by the natural inhibitor of this enzyme isolated from pea sprout homogenates (Grabarek *et al.*, 1965).

Our results concerning the effect of metal ions on plant ASA-synthetase and ASA-lyase are in agreement with those of Ratner & Petrack (1951) and Ratner, Anslow & Petrack (1953) for mammalian preparations, except those for Co^{2+} and Mn^{2+}. Cobalt ions inhibited more effectively the plant enzyme than the mammalian one, and with Mn^{2+} the reverse was true. PCMB inhibited ASA-lyase much less than ASA-synthetase, by 20% at 3.75 mM and 70% at 1.25 mM concentration, respectively. KCN had a similar effect. ASA-synthetase was inhibited at 6.25 mM--KCN concentration by 35% and at 25 mM by 62%, whereas ASA-lyase at the latter KCN concentration was inhibited only by 19%.

Our failure in separating ASA-synthetase from ASA-lyase in wheat sprouts using the methods for liver enzymes (Ratner & Pappas, 1949a,b; Ratner, 1955) and other procedures mentioned in the text could be due to differences in cell protein composition as well as in electrical charge of animal and plant proteins. This is probable since plant enzyme preparation heated for 1 min. at 60° lost all its activity, whereas animal preparation (Rochovansky & Ratner, 1961) heated for 3 min. in the same conditions at 61° showed threefold purification.

Summarizing our results, we can conclude that synthesis of arginine from citrulline and aspartate in higher plants has the same enzymic mechanism as in mammals and micro-organisms. The kinetic properties of ASA-lyase and ASA-synthetase were found to be the same in plants as in other organisms.

It is worth noting that apart from the "classical" path of arginine synthesis from citrulline and aspartate, we have found in wheat sprouts another path for the synthesis of this amino acid from carbamoylaspartate and ornithine (Kleczkowski & Grabarek-Bralczyk, 1968). This new activity in rat-liver preparations was about ten times lower than in plant material.

REFERENCES

Brown G. W., Jr. & Cohen P. P. (1959). *J. Biol. Chem.* **234**, 1769.
Brown G. W., Jr., Brown W. R. & Cohen P. P. (1959). *J. Biol. Chem.* **234**, 1775.
Buraczewski S., Kleczkowski K. & Reifer I. (1960). *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **8**, 93.

Cimino P., Venuta S. & Salvatore F. (1966). *Boll. Soc. Ital. Biol. Sper.* **42**, 2026.

Coleman R. G. & Hegarty M. P. (1957). *Nature* **179**, 376.

Conway E. J. (1947). *Microdiffusion analysis and volumetric error.* Crosby, Lockwood & Son Ltd., London.

De Deken R. H. (1962). *Biochim. Biophys. Res. Commun.* **8**, 462.

Fotyma M. W., Kleczkowski K. & Reifer I. (1961). *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **9**, 61.

Grabarek J., Reifer I. & Kleczkowski K. (1965). *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **13**, 377.

Kasting R. & Delwiche C. C. (1955). *Plant Physiology* **30**, suppl. XXVIII.

Kleczkowski K. (1957). *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **5**, 83.

Kleczkowski K. (1958). *Acta Biochim. Polon.* **5**, 155.

Kleczkowski K. & Grabarek-Bralczyk J. (1968). *Acta Biochim. Polon.* **15**, 283.

Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265.

Ratner S. (1954). *Advanc. Enzymol.* **15**, 319.

Ratner S. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 2, p. 356. Academic Press, New York.

Ratner S. (1962). In *The Enzymes* (P. D. Boyer, H. Lardy & K. Myrbäck, eds.) vol. 6, p. 495. Academic Press, New York, London.

Ratner S. & Pappas A. (1949a). *J. Biol. Chem.* **179**, 1183.

Ratner S. & Pappas A. (1949b). *J. Biol. Chem.* **179**, 1199.

Ratner S. & Petrack B. (1951). *J. Biol. Chem.* **191**, 693.

Ratner S. & Petrack B. (1953a). *J. Biol. Chem.* **200**, 161.

Ratner S. & Petrack B. (1953b). *J. Biol. Chem.* **200**, 175.

Ratner S., Anslow W. P., Jr. & Petrack B. (1953). *J. Biol. Chem.* **204**, 115.

Reifer I. & Buraczewska L. (1958). *Acta Biochim. Polon.* **5**, 361.

Rochovansky O. & Ratner S. (1961). *J. Biol. Chem.* **236**, 2254.

Rochovansky O. & Ratner S. (1967). *J. Biol. Chem.* **242**, 3839.

Severina I. S. (1962). *Biokhimija* **27**, 943.

Wampler D. E. & Fairley J. L. (1967). *Arch. Biochem. Biophys.* **121**, 580.

SYNTEZA ARGININY Z CYTRULINY I ASPARAGINIANU W PREPARATACH Z KIEŁKÓW PSZENICY

Streszczenie

1. W kiełkach pszenicy stwierdzono syntezę argininy z cytruliny i asparaginianu w obecności ATP i Mg^{2+}, katalizowaną przez syntetazę i liazę argininobursztynianową.

2. Wyizolowano i 16-krotnie oczyszczono z ekstraktów proszków acetonowych kiełków pszenicy preparat enzymatyczny zawierający obie aktywności syntezy argininy.

3. Przebadano własności i kinetykę syntetazy oraz liazy argininobursztynianowej i stwierdzono, że oba enzymy nie różnią się zasadniczo od enzymów zwierzęcych.

RENATA A. ZVYAGILSKAYA*, KRYSTYNA BOGUCKA and L. WOJTCZAK

ACCUMULATION OF AZIDE IN MITOCHONDRIA
AND THE EFFECT OF AZIDE ON ENERGY METABOLISM

Department of Biochemistry, Nencki Institute of Experimental Biology, ul. Pasteura 3, Warszawa 22

1. Respiring rat-liver mitochondria accumulate inorganic azide. This process is prevented by 2,4-dinitrophenol and arsenate. 2. Accumulation of calcium by rat-liver and bovine heart mitochondria is more strongly inhibited by sodium azide when the energy is provided by hydrolysis of ATP than when it is supplied by the oxidation of respiratory substrates. 3. Sodium azide inhibits the reverse electron transport from succinate to NAD^+ in rat-liver mitochondria. 4. These results indicate that, apart from being accumulated in mitochondria, azide is an inhibitor of the energy transfer and an uncoupler of energy conservation in mitochondria.

The action of inorganic azide on respiratory and energy metabolism of micro--organisms and animal and plant tissues has long been studied. Three kinds of effects have been observed: (1) inhibition of cytochrome oxidase (Keilin, 1939), (2) uncoupling of respiration from energy conservation (Loomis & Lipmann, 1949; Judah, 1951; Slater, 1955), and (3) inhibition of the energy conservation mechanism (Robertson & Boyer, 1955). Bogucka & Wojtczak (1966a,b) made an attempt to reconcile these different views and postulated a dual effect of azide on the energy metabolism, i.e. the uncoupling and the inhibition of the energy transfer. These two effects may either concern the same coupling site, or sites, or, more likely, occur separately at different sites, the uncoupling at the first coupling site and the inhibition of energy transfer at the two other sites. In the light of the chemical coupling theory (cf. Slater, 1966) the mechanism of this dual effect may be explained by assuming the formation by azide of an unstable complex with a high-energy intermediate of oxidative phosphorylation at one coupling site and a stable complex at another site (or sites). Wilson & Chance (1967) and Wilson (1967) have indeed presented spectrophotometric indications for the energy-dependent formation of a complex of azide with cytochrome *a*.

As shown previously (Bogucka & Wojtczak, 1966a,b), the dual effect of azide on energy metabolism of isolated liver mitochondria is manifested by (*a*) stimulation

* Present address: Bakh Institute of Biochemistry, Leninski prospect 33, Moscow, USSR

of state 4 (Chance & Williams, 1955) respiration with NAD-linked substrates but not with succinate or ascorbate+tetramethyl-p-phenylenediamine, (b) inhibition of state 3 (Chance & Williams, 1955) respiration with all substrates examined, this inhibition being partly reversed by uncouplers, (c) stimulation of the latent (Mg^{2+}-activated) ATPase, and (d) inhibition of the 2,4-dinitrophenol-stimulated ATPase. The inhibition of the ATP - P_i exchange reaction and lowering of the P/O ratio can be explained by either the uncoupling or the inhibition of energy transfer. The inhibition by azide of the energy transfer (effects b and d) is thus similar to the effect of oligomycin (Lardy, Johnson & McMurray, 1958; Huijing & Slater, 1961), and it has been postulated (Bogucka & Wojtczak, 1966b) that the mechanism of action of these two compounds may be similar. However, Palmieri & Klingenberg (1967) have recently postulated that the apparent oligomycin-like effect of azide on mitochondrial respiration can be explained exclusively on the basis of its inhibitory effect on cytochrome oxidase, assuming that azide is accumulated in mitochondria by an energy-dependent mechanism. Uncoupling of oxidative phosphorylation would block this energy-dependent accumulation and release the accumulated azide resulting in the reversal of the inhibition of oxidation. Palmieri & Klingenberg (1967) provided indirect evidence for such accumulation of azide in mitochondria.

The present investigation provides direct evidence for the energy-dependent accumulation of azide in mitochondria, but shows that, apart from its inhibitory effect on cytochrome oxidase, azide does also inhibit energy transfer in oxidative phosphorylation in a way similar to that manifested by oligomycin.

METHODS

Rat-liver mitochondria were isolated as described by Hogeboom (1955) and bovine heart sarcosomes as described by Löw & Vallin (1963).

Oxygen uptake was measured polarographically using a Clark type oxygen electrode.

Calcium uptake by rat-liver mitochondria was measured using $^{45}CaCl_2$ as described by Lehninger, Rossi & Greenawalt (1963). The incubation was carried out for 15 min. at 20°, then the mixture was rapidly chilled to 0° and centrifuged at 17 000 g. The pellet was washed with cold 0.25 M-sucrose and the radioactivity in mitochondria was counted with a thin-window Geiger counter. Calcium accumulated in bovine heart sarcosomes was measured by a similar procedure, except that ATP was completely omitted from the medium in the case when energy was provided by succinate oxidation (incubation medium, see legend to Fig. 4).

Redox changes of intramitochondrial nicotinamide nucleotides were recorded fluorimetrically.

Accumulation of azide in mitochondria was investigated either by membrane filtration or using the silicone-oil filtration procedure (Klingenberg, Pfaff & Kröger, 1964; Pfaff, 1965). In the latter procedure the centrifuge tubes contained, from the bottom up, the following four layers: (1) 0.5 M-perchloric acid in 0.4 M-sucrose;

(2) 2.0 ml. silicone oil (AR 100+AR 150, 1:1 v/v; Wacker-Chemie GmbH, München, German Federal Republic); (3) 2.0 ml. incubation medium containing 230 mM-sucrose, 10 mM-KCl, 3 mM-MgCl$_2$, 20 mM-tris-Cl⁻(pH 7.4), 1 mM-EDTA, 2 mM--respiratory substrate, 1 μM-rotenone (only when succinate was the substrate) and 2 mM-sodium azide; and (4) 0.5 ml. mitochondria suspension in 125 mM-KCl+ +20 mM-tris-Cl⁻(pH 7.4), corresponding to 10 - 15 mg. protein. The tubes were centrifuged in SW39 rotor of Spinco preparative ultracentrifuge increasing the speed from 0 to 8000 rev./min. (0 to 5000 g average) during 4 min. followed by 5 min. at 20 000 rev./min. (32 000 g average). The temperature was 20°. When the membrane filtration was used, mitochondria (10 - 15 mg. protein) were incubated for 1 min at 20° in the incubation medium (3), filtered through a double layer of filters (Sartorius GmbH, Göttingen, German Federal Republic) of pore sizes of 0.8 μ and 0.45 μ respectively, rapidly washed with cold 0.25 M-sucrose, and extracted with 0.7 M-perchloric acid.

The determination of azide was based on the observation that azide interferes with the colorimetric determination of hydroxylamine by the method described by Csáky (1948). This interference is caused by the interaction between azide and nitrous acid which is formed from hydroxylamine. The procedure was as follows: 0.4 ml. of the sample (perchloric acid extract of mitochondria neutralized with potassium bicarbonate) was added to 0.1 ml. of 0.4 mM-hydroxylamine, and to this mixture 0.2 ml. of the sulphanilic acid reagent and 0.1 ml. of iodine solution were added. After 4 to 5 min. the excess of iodine was reduced by the addition of 0.2 ml.
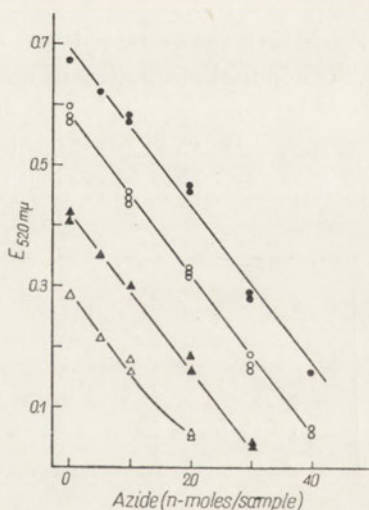

Fig. 1. Colorimetric determination of sodium azide. Calibration curves. Hydroxylamine: 20 n-moles (△), 30 n-moles (▲), 40 n-moles (○), 50 n-moles (●). Total volume 1.2 ml., other conditions as under Methods.

of 2% sodium arsenite, and 0.2 ml. of α-naphthylamine solution was added subsequently. After 25 - 30 min. standing at room temperature the extinction at 520 mμ was measured against a blank containing all reagents except hydroxylamine. Parallel measurements were made with samples: (a) containing the reagents plus hydroxylamine but not containing azide, (b) containing all reagents (including hydro-

xylamine) plus mitochondrial extract but not containing azide, and (c) containing a known quantity of azide. It appeared that the decrease of the extinction was linear with increasing amounts of azide (Fig. 1). By this procedure as little as 5 n--moles azide could be easily determined. The amount of azide was calculated from the equation:

$$x = \frac{E_b - E_x}{E_a - E_c}\, c$$

where E_a, E_b, E_c and E_x are extinctions of sample (a), (b), (c) and the unknown respectively, and c is the amount of azide in sample (c). Reagents: 0.4 mM-hydroxylamine, freshly diluted from the 4 M stock solution; sulphanilic acid reagent, 1 g. sulphanilic acid dissolved in 100 ml. glacial acetic acid; α-naphthylamine solution, 300 mg. α-naphthylamine dissolved in 100 ml. 30% acetic acid. This method will be described in detail elsewhere (Zvyagilskaya & Wojtczak, in preparation).

Mitochondrial protein was determined by the biuret method (Gornall, Bardawill & David, 1949) after solubilizing mitochondria in deoxycholate solution.

Sodium azide was the product of Merck A.G. (Darmstadt, German Federal Republic).

RESULTS

Effect of azide on mitochondrial respiration

It has been shown by Palmieri & Klingenberg (1967) that the highest sensitivity of mitochondrial respiration to azide occurs in the ion pumping state, in the presence of valinomycin $+K^+$. This observation was confirmed in the present investigation (Fig. 2). Contrary to this, gramicidin did not potentiate the inhibitory effect of azide. This is compatible with the fact that gramicidin uncouples the
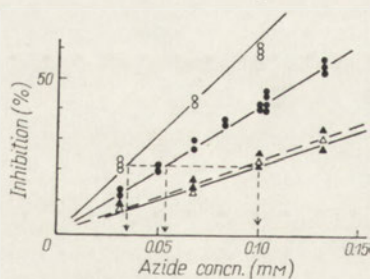

Fig. 2. Inhibition by azide of succinate oxidation by rat-liver mitochondria in various energetic states. Incubation medium: 250 mM-sucrose, 20 mM-tris-HCl (pH 7.4), 10 mM-KCl, 10 mM-Na-succinate, 1 μM-rotenone, 2 mg. mitochondrial protein and other additions as indicated below. Total volume 3.0 ml.; temperature 25°. ○— ○, 0.3 μg. valinomycin; ●— ●, 2 mM-P_i+1 mM-ADP; ▲— ▲ 0.1 mM-2,4-dinitrophenol; △- - -△, 0.5 μg. gramicidin. Dashed arrows indicate concentrations of azide giving the same degree of inhibition in various energetic states of mitochondria.

energy transfer (Harris, Höfer & Pressman, 1967) while valinomycin does not. Assuming that the degree of the inhibition of respiration is an index of intramitochondrial concentration of azide and that in the uncoupled state intramitochondrial and extramitochondrial concentrations of azide are equal, it can be calculated that,

at low azide concentrations where there is a linear relationship between the degree of inhibition and the concentration of azide, a threefold and a twofold accumulation of azide occur in ion pumping state and in state 3 respectively (Fig. 2). A somewhat greater accumulation can be calculated from experiments of Palmieri & Klingenberg (1967) (see Fig. 3 in their paper).

Accumulation of azide in mitochondria

Direct evidence for azide accumulation in mitochondria was obtained by chemical determination. When mitochondria were briefly incubated in a medium containing 2 mM-sodium azide and a respiratory substrate an accumulation of azide in mitochondria was found. Assuming that the amount of intramitochondrial water is equal to the amount of protein, a twofold increase of azide concentration inside mitochondria could be calculated under conditions of states 4 and 3 but only a small increase if any under uncoupled conditions (Table 1, Expt. 1). Expt. 2 of Table 1, carried out using a different filtration procedure, confirms these results, although corresponding values of azide accumulation are here somewhat lower, presumably due to a partial elution during a brief washing of the filters with sucrose solution. Yet, this error should be similar for all samples and therefore should not obscure the results. Expt. 2 also shows an important observation that azide accumulation is diminished not only by 2,4-dinitrophenol but also by arsenate.

Results similar to those shown in Table 1 with succinate as the respiratory substrate were also obtained using β-hydroxybutyrate and ascorbate+tetramethyl-p--phenylenediamine.

Effect of azide on mitochondrial respiration uncoupled by arsenate

Arsenate presents a good tool to differentiate between the effect of azide as an accumulable anion inhibiting the terminal electron transport on one side and the

Table 1

Accumulation of azide in mitochondria

For experimental conditions see the Methods; the respiratory substrate was succinate.

Expt. no.	Procedure	Additions to the incubation mixture	Concentration of azide in mitochondria (mM)
1	Silicone-oil filtration	P_i, 3 mM	3.8
		P_i, 3 mM+ADP, 1 mM	4.0
		P_i, 3 mM+2,4-dinitrophenol, 0.1 mM	2.8
2	Membrane filtration	None	3.1
		P_i, 1 mM	2.2
		2,4-Dinitrophenol, 0.1 mM	1.5
		Arsenate, 1 mM	1.1

inhibition of energy transfer on the other side. Although arsenate prevents the accumulation of azide in mitochondria (Table 1), it does not release the inhibitory effect of azide on succinate oxidation, this inhibition being, however, released by 2,4-dinitrophenol (Fig. 3). This experiment suggests that a smaller inhibition by azide
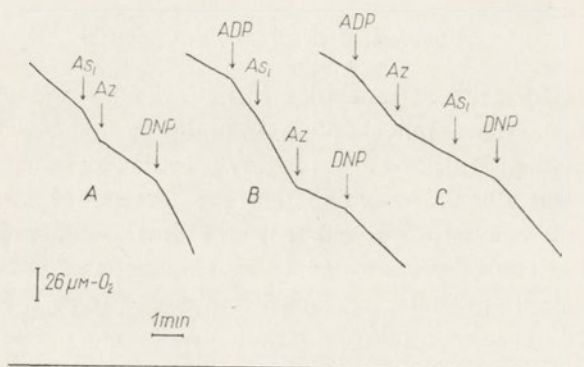

Fig. 3. Effect of azide and arsenate on succinate oxidation. Incubation conditions as in Fig. 2, except that 6 mg. mitochondrial protein is present, and traces B and C contain 1 mM-P_i. Additions: 1 mM-arsenate (As_i), 1 mM-sodium azide (Az), 0.1 mM-2,4-dinitrophenol (DNP), 1 mM-ADP.

of mitochondrial respiration under uncoupled conditions than in state 3 (Bogucka & Wojtczak, 1966b; Wilson & Chance, 1966, 1967; Palmieri & Klingenberg, 1967) cannot be entirely explained by a release by uncouplers of intramitochondrially accumulated azide. On the other hand, the effect of azide on mitochondrial respiration in the presence of arsenate is quite similar to the effect of oligomycin (Estabrook, 1961), which suggests a similar mechanism of action.

Effect of azide on calcium accumulation

It is well known that the energy-dependent accumulation of divalent cations in mitochondria can be driven by either the hydrolysis of ATP or the electron transport (cf. Lehninger, Carafoli & Rossi, 1967). In the first case the accumulation can be inhibited by inhibitors of the energy transfer, e.g. oligomycin, but is insensitive to the inhibitors of the respiratory chain; in the second case it can be blocked by inhibitors of the respiratory chain but is insensitive to oligomycin. Therefore, the accumulation of divalent cations can provide a crucial test system to elucidate whether, apart from the inhibitory action on the respiratory chain, azide has any effect on the energy-transfer system in mitochondria.

Figure 4 shows the effect of azide on calcium accumulation in rat-liver mitochondria. It is evident that the inhibition is much greater when ATP is the energy donor than when the energy is provided by the oxidation of succinate (or other respiratory substrates). Similar results were obtained with bovine heart sarcosomes.

Fig. 4. Calcium accumulation in rat-liver mitochondria. Incubation medium: 80 mM-NaCl, 10 mM-tris-HCl (pH 7.4), 9 mM-MgCl$_2$, 3 mM-P$_i$, 2 mM-^{45}CaCl$_2$ (10^5 counts/min.) and 3 mg. mitochondrial protein; total volume 1.0 ml. ●— ●, 10 mM-Na-succinate, 1 μM-rotenone and 2 mM-ATP; ○— ○, 15 mM-ATP; ▲, 10 mM-Na-succinate, 1 μM-rotenone, 2 mM-ATP and 2 mM-KCN; △, 15 mM-ATP and 4 μg. oligomycin.
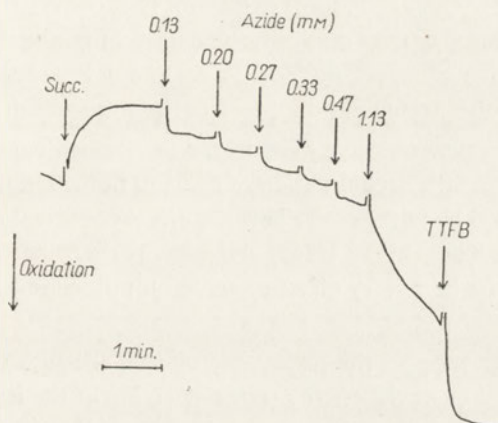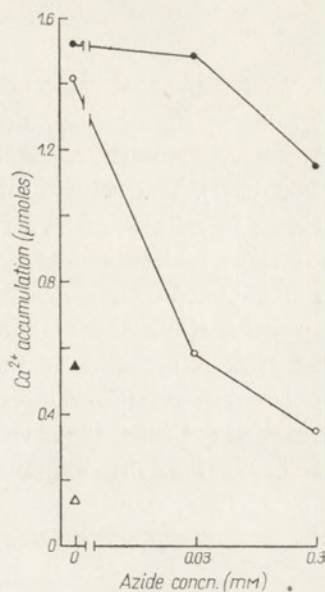

Fig. 5. Effect of azide on the reverse electron transport. Redox changes of mitochondrial nicotinamide nucleotides. Incubation medium: 120 mM-KCl, 20 mM-tris-HCl (pH 7.4), 3 mM-MgCl$_2$, and 4.0 mg. mitochondrial protein. Total volume 3.0 ml., temperature 20°. Additions: 5 mM-Na-succinate (Succ.); sodium azide, final concentration as indicated; 0.5 μM-tetrachloro-trifluoro-methyl-benzimidazole (TTFB).

Effect of azide on the reverse electron transport and redox states of nicotinamide nucleotides

In view of the different postulated effect of azide on the respiratory and energy-conserving mechanisms it was of interest to investigate its effect on the reverse electron transport and the redox state of NAD(P) in mitochondria. Fig. 5 shows that small concentrations of azide partly decrease the degree of NAD(P) reduction by succinate, most likely due to a partial uncoupling. When the concentration of azide is increased a complete oxidation of NAD(P) occurs. .

DISCUSSION

We have shown previously (Bogucka & Wojtczak, 1966a,b) that the inhibition by azide of state 3 respiration can be largely reversed by uncouplers of oxidative phosphorylation. The same effect has also been demonstrated by Wilson & Chance (1966, 1967). This finding, together with some other observations, e.g. on the effect of azide on mitochondrial ATPase, led us (Bogucka & Wojtczak, 1966b) to postulate that azide inhibits the energy transfer system of mitochondria in a way similar to that exerted by oligomycin (Lardy *et al.*, 1958; Huijing & Slater, 1961). However, Palmieri & Klingenberg (1967) proposed another explanation. They have postulated that azide is accumulated in mitochondria in an energy-requiring process similar to that responsible for the energy-dependent accumulation of anionic substrates (Chappell & Crofts, 1966; Harris, van Dam & Pressman, 1967; Quagliariello & Palmieri, 1968). It then inhibits the respiration at the level of cytochrome oxidase (Keilin, 1939; Yonetani & Ray, 1965). Uncouplers of oxidative phosphorylation release the accumulated azide and in this way they diminish the degree of inhibition. However, because of lack of a sensitive chemical method Palmieri & Klingenberg were unable to demonstrate directly the accumulation of azide in mitochondria.

In the present investigation, using a new sensitive method for the determination of azide, we were able to demonstrate that the accumulation of azide in mitochondria does indeed occur and that it is abolished by uncouplers. This is a direct confirmation of the postulation made by Palmieri & Klingenberg (1967) and may, perhaps partly, explain the reversal by uncouplers of the inhibitory effect of azide on mitochondrial respiration.

There are, however, data which also support the earlier postulation (Bogucka & Wojtczak, 1966b) that azide is an inhibitor of the energy transfer, acting at the level of high-energy intermediates of the energy-coupling mechanism (an oligomycin--like effect). Earlier experiments of Brierley, Murer & Bachmann (1964) with heart sarcosomes and of Hodges & Elzam (1967) with plant mitochondria as well as the results of the present investigation obtained with both liver and heart mitochondria clearly indicate that azide is a much stronger inhibitor of calcium accumulation when ATP is the energy donor than when energy is provided by the oxidation of respiratory substrates. If azide were only the inhibitor of the respiratory chain, the effect on calcium accumulation would be quite opposite. Thus, the effect of azide resembles in this respect that of oligomycin, which suggests that the mechanism of action of these two compounds may be similar. Furthermore, a strong inhibitory action of azide on the dinitrophenol-stimulated mitochondrial ATPase (Robertson & Boyer, 1955; Swanson, 1956; Myers & Slater, 1957; Siekevitz, Löw, Ernster & Lindberg, 1958; Bogucka & Wojtczak, 1966b) and the ATPase activity of coupling factor F_1 (Pullman, Penefsky, Datta & Racker, 1960; Penefsky, Pullman, Datta & Racker, 1960) also points to the inhibition of energy transfer. A similar conclusion has also been recently expressed by Vigers & Ziegler (1968) on the basis of their study on the effect of azide on soluble mitochondrial ATPase preparation.

Another indication that azide acts as an inhibitor of the energy transfer is provided by experiments with arsenate. Their results (Fig. 3) cannot be easily explained in terms of azide accumulation in mitochondria, but they rather suggest that azide has a specific effect on the energy transfer mechanism similar to that of oligomycin. As it is well known (Estabrook, 1961; Ernster, Lee & Janda, 1967), the mechanism of uncoupling by arsenate differs from that by dinitrophenol and other typical uncouplers.

The present investigation also provides a further example of the uncoupling effect of azide (Bogucka & Wojtczak, 1966b), namely the inhibition of the reverse electron transport, as manifested by a partial oxidation of mitochondrial nicotinamide nucleotides. This effect is quite different than it would be expected from the oligomycin-like action of azide and from its inhibitory effect on cytochrome oxidase. Under experimental conditions of Fig. 5 oligomycin would have no effect on the reverse electron transport, and the inhibition of the terminal electron transport, e.g. by cyanide or anaerobiosis, would prevent the oxidation of nicotinamide nucleotides. Yet, it seems unlikely that the accumulation of azide by mitochondria would also be responsible for its uncoupling effect, as suggested by Palmieri & Klingenberg (1967). First, because the accumulation quickly attains its climax and therefore is not likely to be responsible for a permanent energy drain; and secondly, because other salts of weak acids with similar pK, e.g. acetate, which are accumulated in mitochondria at the expense of energy (Chappell & Crofts, 1966) do not act as uncouplers. Thus, the uncoupling effect of sodium azide is most likely independent of its accumulation in mitochondria. It is noteworthy that some organic derivatives of azide, e.g. amyl azide, are "pure" uncouplers (Bogucka, Erecińska & Wojtczak, in preparation). Sodium azide differs, however, from other uncouplers, such as 2,4-dinitrophenol or dicoumarol, in that it does not increase proton conductance of artificial phospholipid membranes (Zvyagilskaya & Liberman, unpublished results).

In conclusion, the present investigation shows that azide can be accumulated in mitochondria by an energy-dependent mechanism, but independently it is also an inhibitor of the energy transfer, analogous in its action to oligomycin. Finally, a true uncoupling of the energy-conserving mechanism is another effect of azide.

REFERENCES

Bogucka K. & Wojtczak L. (1966a). *3rd FEBS Meeting, Warsaw, Abstract Commun.* No. M 43, p. 135.

Bogucka K. & Wojtczak L. (1966b). *Biochim. Biophys. Acta* **122**, 381.

Brierley G. P., Murer E. & Bachmann E. (1964). *Arch. Biochem. Biophys.* **105**, 89.

Chance B. & Williams G. R. (1955). *Nature* **176**, 250.

Chappell J. B. & Crofts A. R. (1966). In *Regulation of Metabolic Processes in Mitochondria* (J. M. Tager, S. Papa, E. Quagliariello & E. C. Slater, eds.). p. 293. Biochim. Biophys. Acta Library, vol **7**, Elsevier, Amsterdam.

Csáky T. Z. (1948). *Acta Chem. Scand.* **2**, 450.

Ernster L., Lee C. P. & Janda S. (1967). In *Biochemistry of Mitochondria* (E. C. Slater, Z. Kaniuga & L. Wojtczak, eds.), p. 29. Academic Press and P.W.N., London and Warszawa.

Estabrook R. W. (1961). *Biochem. Biophys. Res. Commun.* **4**, 89.

Gornall A. G., Bardawill C. J. & David M. M. (1949). *J. Biol. Chem.* **177**, 751.

Harris E. J., van Dam K. & Pressman B. C. (1967). *Nature* **213**, 1126.

Harris E. J. Höfer M. P. & Pressman B. C. (1967). *Biochemistry* **6**, 1348.

Hodges T. K. & Elzam O. E. (1967). *Nature* **215**, 970.

Hogeboom G. H. (1955). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 1, p. 16. Academic Press, New York.

Huijing F. & Slater E. C. (1961). *J. Biochem. (Tokyo)* **49**, 493.

Judah J. D. (1951). *Biochem. J.* **49**, 271.

Keilin D. (1939). *Proc. Roy. Soc. (London), Ser. B*, **121**, 523.

Klingenberg M., Pfaff E. & Kröger A. (1964). In *Rapid Mixing and Sampling Techniques in Biochemistry* (B. Chance, ed.), p. 333. Academic Press, New York.

Lardy H. A., Johnson D. & McMurray W. C. (1958). *Arch. Biochem. Biophys.* **78**, 587.

Lehninger A. L., Carafoli E. & Rossi C. S. (1967). *Advanc. Enzymol.* **29**, 259.

Lehninger A. L., Rossi C. S. & Greenawalt J. W. (1963). *Biochem. Biophys. Res. Commun.* **10**, 444.

Loomis W. F. & Lipmann F. (1949). *J. Biol. Chem.* **179**, 503.

Löw H. & Vallin I. (1963). *Biochim. Biophys. Acta* **69**, 361.

Myers D. K. & Slater E. C. (1957). *Biochem. J.* **67**, 572.

Palmieri F. & Klingenberg M. (1967). *European J. Biochem.* **1**, 439.

Penefsky H. S., Pullman M. E., Datta A. & Racker E. (1960). *J. Biol. Chem.* **235**, 3330.

Pfaff E. (1965). *Thesis.* Philipps-Universität, Marburg/Lahn. German Federal Republic.

Pullman M. E., Penefsky H. S., Datta A. & Racker E. (1960). *J. Biol. Chem.* **235**, 3322.

Quagliariello E. & Palmieri F. (1968). *European J. Biochem.* **4**, 20.

Robertson H. E. & Boyer P. D. (1955). *J. Biol. Chem.* **214**, 295.

Siekevitz P., Löw H., Ernster L. & Lindberg O. (1958). *Biochim. Biophys. Acta* **29**, 378.

Slater E. C. (1955). *Biochem. J.* **59**, 392.

Slater E. C. (1966). In *Regulation of Metabolic Processes in Mitochondria* (J. M. Tager, S. Papa, E. Quagliariello & E. C. Slater, eds.) p. 166. Biochim. Biophys. Acta Library, vol. 7. Elsevier, Amsterdam.

Swanson M. A. (1956). *Biochim. Biophys. Acta* **20**, 85.

Vigers G. A. & Ziegler F. D. (1968). *Biochim. Biophys. Res. Commun.* **30**, 83.

Wilson D. F. (1967). *Biochim. Biophys. Acta* **131**, 431.

Wilson D. F. & Chance B. (1966). *Biochem. Biophys. Res. Commun.* **23**, 751.

Wilson D. F. & Chance B. (1967). *Biochim. Biophys. Acta* **131**, 421.

Yonetani T. & Ray G. S. (1965). *J. Biol. Chem.* **240**, 3392.

AKUMULACJA AZYDKU W MITOCHONDRIACH
I WPŁYW AZYDKU NA PRZEMIANY ENERGETYCZNE

Streszczenie

1. Mitochondria wątroby szczura utleniające substraty oddechowe akumulują nieorganiczny azydek. Akumulacja ta jest hamowana przez 2,4-dwunitrofenol i arsenian.

2. Akumulacja wapnia przez mitochondria wątroby szczura i serca wołu jest silniej hamowana przez azydek sodu, gdy źródłem energii jest hydroliza ATP, niż wówczas gdy energii dostarcza utlenianie substratów oddechowych.

3. Azydek sodu hamuje odwrotny transport elektronów z bursztynianu na NAD^+ zachodzący w mitochondriach wątroby szczura.

4. Wyniki te wskazują, że azydek — niezależnie od tego, że jest akumulowany w mitochondriach — jest również inhibitorem transportu energii w mitochondriach, a także czynnikiem rozprzęgającym oksydacyjną fosforylację.

INA GĄSIOROWSKA, ZOFIA POREMBSKA and IRENA MOCHNACKA

STUDIES ON OX-BRAIN ARGINASE

Department of Physiological Chemistry, Medical School, Krakowskie Przedmieście 26/28, Warszawa 64

1. Arginase from ox brain was isolated and purified about 70-fold. 2. The enzyme has the following properties: two pH optima, at 7.5 and 9.5; K_m 9 mM; molecular weight 120 000. The enzyme is activated by Mn^{2+} ion, inhibited by excess of substrate, and competitively inhibited by ornithine and lysine. 3. The enzyme is located in nuclei and mitochondria. 4. Two arginase isoenzymes were found both in ox brain and rat liver; the isoenzymes did not differ in K_m value and molecular weight.

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1), an enzyme which catalyses the hydrolysis of arginine to urea and ornithine, occurs in ureotelic animals not only in the liver but also in tissues in which the whole ornithine cycle does not occur.

The presence of arginase in rat brain has been demonstrated by Sporn, Dingman, Defalco & Davies (1959a,b), in dog brain by Tomlinson & Westall (1960), in the brain of ox, rat, monkey and man by Ratner, Morell & Caravalho (1960), and in the brain of uricotelic animals (chicken and frog) by Buniatian & Davtian (1965).

It is known from the studies of Mora, Tarrab, Martuscelli & Soberon (1965) that liver arginase of ureotelic vertebrates differs from liver arginase of uricotelic vertebrates (chicken, lizard). The "ureotelic" arginase, as compared with the "uricotelic" enzyme, has a lower molecular weight, an almost 10-fold smaller K_m value, and is inhibited by excess of substrate.

The aim of the present work was to isolate the enzyme from ox brain and to determine whether the arginase of an ureotelic animal but derived from a tissue in which the process of ureogenesis does not occur, is of the "ureotelic" or "uricotelic" type.

MATERIALS AND METHODS

Reagents. Sephadex G-100 (Pharmacia, Uppsala, Sweden), Whatman DEAE--cellulose, DE 11 (Balston Ltd., Maidstone, Kent, England), L-arginine hydrochloride, L-ornithine hydrochloride and L-lysine hydrochloride (Sigma Chem. Co., St. Louis, Mo., U.S.A.), L-glycine (Biuro Obrotu Odczynnikami Chemicznymi,

Gliwice, Poland), L-homoarginine (Koch-Light Lab. Ltd., Colnbrook, Bucks., England), L-canavanine sulphate (Nutr. Biochem. Corp., Cleveland, Ohio, U.S.A.), xanthone (E. Merck A.G., Darmstadt, German Federal Republic), tris (Fluka A.G., Buchs, Switzerland), ninhydrin (Politechnika Śląska, Gliwice, Poland), crystalline bovine serum albumin (Pentox Incorp., Kankakae, U.S.A.), ovalbumin, human albumin and γ-globulin (Warsaw Serum and Vaccine Plant, Warszawa, Poland). γ-Guanidinobutyric acid was prepared after Schütte (1943).

Homogenate and acetone-dried powder from brain tissue. Ox brain was isolated in the slaughterhouse immediately after killing of the animal, transported in ice and kept at -10°. After thawing, the *pia matter* was removed and the tissue was homogenized at 1900 rev./min., twice for 2 min., with 3 volumes of 0.05 M-$MnCl_2$ - - 0.1 M-KCl - 0.01 M-tris-HCl buffer, pH 7.5, in a glass Potter-type homogenizer provided with a teflon pestle. The homogenate was placed in an ice-bath for 30 min. and gently stirred. Then it was centrifuged for 15 min. at 5000 rev./min.; to the supernatant, 1.5 volumes of acetone (-10°) was added slowly with constant stirring. The sediment was centrifuged at -10° and dried at room temperature for 60 min. The acetone-dried powder was stored in the refrigerator in a desiccator. For the experiments, 1 g. of the powder was treated with 10 ml. of 0.01 M-tris-HCl buffer pH 7.5; after 15 min. with stirring, the extraction was repeated, and the combined supernatants were used.

Subcellular fractions. These were prepared according to Hogeboom (1960) except that for homogenization, instead of 0.25 M-sucrose, a 0.32 M-sucrose solution was applied as suggested for the brain by Whittaker, Michaelson & Kirkland (1964). The individual fractions were suspended in 0.01 M-tris-HCl buffer, pH 7.5, and frozen. On the next day, the samples were thawed and used for the assay of enzyme activity.

Enzyme assay. The activity of arginase was determined in 1 ml. of a mixture containing: the enzyme preparation, 30 μmoles of L-arginine, 5 μmoles of $MnCl_2$ and 50 μmoles of glycine buffer, pH 9.5. After 15 min. incubation at 37°, the reaction was stopped by placing the samples for 7 min. in a boiling-water bath. The precipitated sediment was centrifuged off and in the supernatant ornithine was determined by the method of Chinard (1952), or urea by the xanthydrol method of Engel & Engel (1947). In either case the same results were obtained. The activity of arginase was expressed as mμmoles of ornithine or urea formed per minute.

Protein determination. The method of Lowry, Rosebrough, Farr & Randall (1951) was employed, with bovine serum albumin as standard. In the course of purification of the enzyme, the concentration of protein was determined by measuring extinction at 260 and 280 mμ according to Warburg & Christian (1941).

Determination of molecular weight. This was performed according to Andrews (1964) by gel filtration on Sephadex G-100.

RESULTS

Activity of extracts from acetone-dried brain tissue

The specific activity of arginase was ten times as high in extracts from acetone-
-dried powder as in extracts from fresh tissue. The extracts from acetone-dried
powder kept at -10° remained active for several weeks. Two- or three-fold freezing
and thawing did not cause a decrease of enzyme activity. At a temperature of 4°
at pH 7 - 9 the activity was unchanged for a few days only. Heating for 5 min. at
pH 7.5 at 50 - 60° caused a 20% decrease of activity, whereas heating at tempera-
tures exceeding 70° inactivated the enzyme by 80% (Fig. 1).
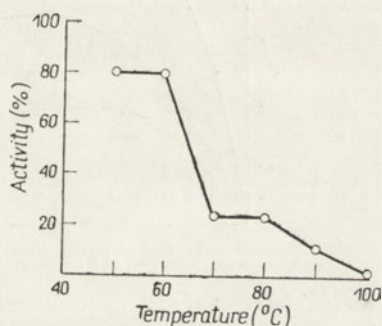
Fig. 1. The effect of temperature on the activity of ar-
ginase from extract of acetone-dried brain tissue. The
tris-HCl extract, pH 7.5, was heated for 5 min. at the
indicated temperature, then rapidly cooled, and the
activity was determined at 37° as described in Methods.
The value obtained for the unheated extract was taken
as 100.

The rate of arginine hydrolysis was linear with protein concentration between
0.5 and at least 2 g. of protein (Fig. 2a), and with 0.5 mg. protein it was linear with
time for 1 hr. (Fig. 2b). Two pH optima for arginine activity were observed: at
pH 7 and 9 - 9.5 (Fig. 2c).
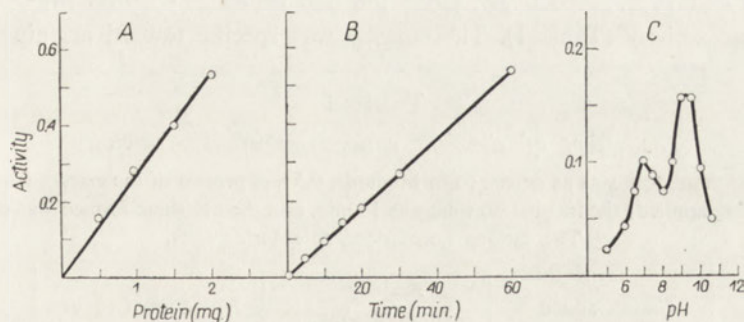
Fig. 2. The effect of (*A*), protein concentration, (*B*), incubation time and (*C*), pH value, on the
activity of arginase in extract from acetone-dried brain. Conditions: for expt. *A* the incubation
mixture contained in 1 ml.: 30 μmoles of arginine, 5 μmoles of $MnCl_2$, 50 μmoles of glycine buffer,
pH 9.5; incubation time 15 min.; for expt. *B* the conditions were the same except that 0.5 mg.
of protein was applied, and in expt. *C* for pH values 4 - 5 acetate buffer, for pH 6 - 8 tris-HCl and
for pH 9 - 10 glycine buffer were used. The activity is expressed as μmoles of ornithine formed
per sample.

The K_m value for brain arginase calculated from the Lineweaver-Burk plot was 9 mM (the range being from 8 to 12 mM), and the enzyme was inhibited by excess of substrate. Ornithine and lysine proved to be competitive inhibitors of brain arginase, lysine being the stronger inhibitor (Fig. 3).
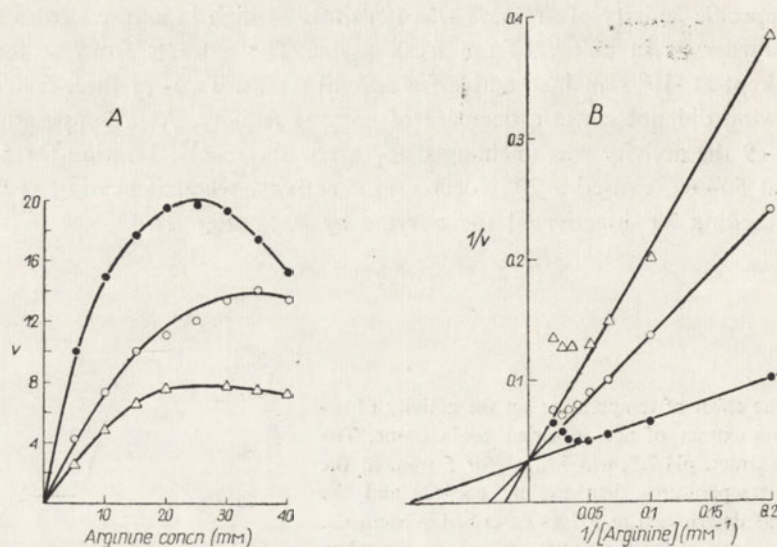
Fig. 3. The effect of substrate concentration on arginase activity in extract from acetone-dried brain. The incubation mixture contained in 1 ml.: 0.5 mg. of protein, 5 μmoles of $MnCl_2$ and 50 μmoles of glycine buffer, pH 9.5. After 30 min. incubation at 37°, the enzyme activity was determined by measuring the urea formed. A: (●), uninhibited reaction; (○), after addition of 10 μmoles of ornithine; (△), after addition of 10 μmoles of lysine. B: Lineweaver-Burk plot of the same data. v is expressed as mμmoles of urea/mg. protein/min.

Mn^{2+}, Cd^{2+} and Ni^{2+} ions at a concentration of 5 mM activated the arginase by 42, 30 and 18%, respectively, Co^{2+} ion had no effect whereas Mg^{2+} inhibited the enzyme activity (Table 1). The enzyme was specific toward arginine and did

Table 1

The effect of divalent cations on arginase activity

The incubation mixture was as described in Methods, 0.5 mg. protein of the extract from acetone-powder being applied; the incubation time was 15 min. and the ornithine formed was determined. The cations were added as chloride salts.

Cation added (5 mM)	Activity (mμmoles/mg. protein/min.)	Activation (+) or inhibition (—) (%)
None	14.1	—
Mn^{2+}	20.0	+42
Cd^{2+}	18.3	+29
Ni^{2+}	16.6	+18
Co^{2+}	13.6	+3
Mg^{2+}	10.6	—25

not catalyse the formation of urea from other guanidine derivatives such as L-homo-arginine, L-canavanine and γ-guanidinobutyrate.

Subcellular distribution of arginase

Distribution of brain arginase in subcellular fractions is shown in Table 2. The enzyme was found mainly in the nuclear and mitochondrial fractions, and only 12% of the activity was in the two other fractions.

Table 2

Localization of arginase in subcellular fractions of ox brain

Arginase activity was determined as described in Methods. Protein, after being precipitated with trichloroacetic acid and dissolved in 1 N-NaOH, was estimated by the method of Lowry *et al.* (1951).

Preparation	Protein (mg.)	Activity		
		specific (mμmoles/ /min./mg. protein)	total (mμmoles/ /min.)	distribution (%)
Homogenate	300	3	900	100
Nuclei fraction, 600 *g*	120	2.5	300	33.3
Mitochondrial fraction, 15 000 *g*	90	4.6	420	46.6
Microsomal fraction, 105 000 *g*	24	2.1	51.6	5.7
Supernatant	50	1.1	58.3	6.5

Table 3

Purification of arginase from ox brain

For the purification procedure, 50 g. of the brain was taken. The details of purification are described in the text.

Fraction	Volume (ml.)	Total protein (mg.)	Activity		Purification factor
			total (mμmoles/ /min.)	specific (mμmoles/ /min./mg. protein)	
Homogenate	140	2100	4833	2.3	—
Extract	110	1100	5500	5.0	2
Extract from acetone- -powder	30	240	5200	21.6	10
Ppt. at 0.25 - 0.7 (NH$_4$)$_2$SO$_4$ sat.	5	80	3600	45	20
DEAE-cellulose chroma- tography, peak *II*	50	12.5	1708	136.6	60
Ppt. at 0 - 0.9 (NH$_4$)$_2$SO$_4$ sat.	2	8	1200	150	68

Isolation and purification of arginase from ox brain

The procedures used for isolation of the enzyme from other tissues: heating at 50 - 60° and fractionation with isopropanol (Greenberg, Bagot & Roholt, 1956; Bach & Killip, 1961; Bach, Hawkins & Swaine, 1963), were ineffective for isolation of brain arginase present in the extract from acetone-dried tissue. Similarly, the CM-cellulose chromatography used by Schimke (1962) could not be applied because the enzyme was not adsorbed on the column.

In the adopted procedure, all steps of purification were carried out in a cold room at about 4°. The results are presented in Table 3.

To the extract from acetone-dried powder from 50 g. of brain tissue, ammonium sulphate was added at pH 7, to 0.25 saturation. After 1 hr. the inactive sediment was centrifuged off and the supernatant brought to 0.75 saturation. After 1 hr. the sediment was collected by centrifugation, dissolved in 5 ml. of 5 mM-tris-HCl buffer, pH 8.3, and dialysed overnight against 5 litres of the same buffer. The dialysed preparation was applied to a DEAE-cellulose column equilibrated with 5 mM--tris-HCl buffer, pH 8.3, and eluted with 30 ml. of the same buffer and then with a linear KCl concentration gradient (up to 0.3 M). The chromatography gave two active peaks (Fig. 4). Peak *I*, eluted with the buffer, contained about 30% of the total recovered enzyme activity. The bulk of arginase emerged as peak *II* at about 0.125 M-KCl concentration.
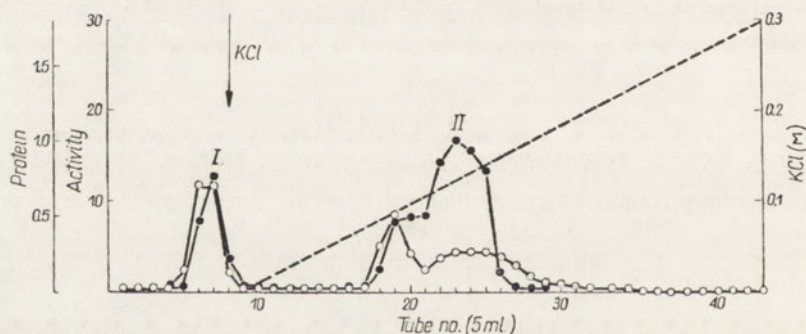

Fig. 4. Chromatography of brain arginase on DEAE-cellulose. The preparation of arginase precipitated at 0.25 - 0.7 $(NH_4)_2SO_4$ saturation and dialysed, 80 mg. of protein in 5 ml. of 5 mM-tris--HCl buffer, pH 8.3, was applied to a column (1×20 cm.) of DEAE-cellulose equilibrated with 5 mM-tris-HCl buffer, pH 8.3. Protein was eluted with 30 ml. of the equilibration buffer, and then with a KCl concentration gradient up to 0.3 M. The flow rate was 16 - 18 ml./hr.; fractions of 5 ml. were collected. (○), Protein (mg./ml.); (●), arginase activity (mμmoles of ornithine/min./ml. of eluate); (— — —), KCl concentration gradient.

The eluates from tubes no. 21 - 25 were pooled and protein was precipitated by adding ammonium sulphate to 0.9 saturation. The centrifuged sediment was dissolved in 2 ml. of 5 mM-tris-HCl buffer, pH 7.5, and dialysed against 3 litres of the same buffer. The slight sediment formed on dialysis was removed by centri-

fugation. The obtained enzyme preparation had a specific activity of 150 mμmoles/
/mın./mg. protein and was purified 70-fold in relation to the homogenate.

This arginase preparation containing about 4 mg. of protein per 1 ml., kept
at -10° at pH 7.5 lost after the first thawing 20% of activity, and was completely
inactivated by three cycles of freezing and thawing. However, the enzyme preci-
pitated by 0.9 ammonium sulphate saturation retained its activity for about two
weeks even at 4°.

The partially purified arginase preparation had the same two pH optima as
were found in the crude extract from acetone-powder (cf. Fig. 2c). The K_m value
was also of the same order and amounted to 8 - 12 mM.

The approximate molecular weight determined by gel filtration on a Sephadex
G-100 column was 120 000 (Fig. 5). The same value was obtained for the arginase
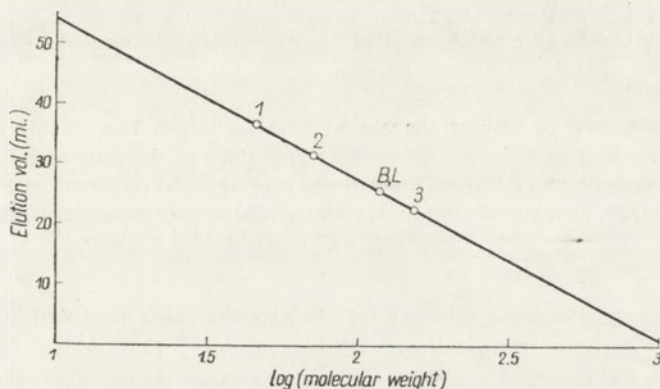

Fig. 5. Determination of the molecular weight of ox-brain arginase by Sephadex G-100 gel filtration.
The enzyme preparation purified about 70-fold (1 ml., containing 6 mg. of protein) was applied
to a column (2×37 cm.), and from the elution volume the molecular weight was determined (An-
drews, 1964). Protein standards: 1, ovalbumin. mol. wt. 45 000; 2, human albumin, mol. wt. 69 000;
3, human γ-globulin, mol. wt. 150 000; B, ox-brain arginase; L, rat-liver arginase, obtained by
the method of Bach & Killip (1961).

isolated from rat liver by the method of Bach & Killip (1961). The liver preparation,
which was purified about 50-fold, when applied to the column together with the
brain arginase, emerged at the same elution volume.

Isoenzymes of arginase from brain and liver

The chromatography of the brain arginase preparation on DEAE-cellulose
column gave two active fractions (see Fig. 4). The smaller arginase fraction (peak I)
was eluted with the buffer, the second fraction was adsorbed on the column and
eluted with a KCl concentration gradient at about 0.125 M. The two isoenzymes
had the same K_m value (about 9 mM) but differed in stability. The first fraction
was inactivated by heating, and at 0° and room temperature it was inactivated more
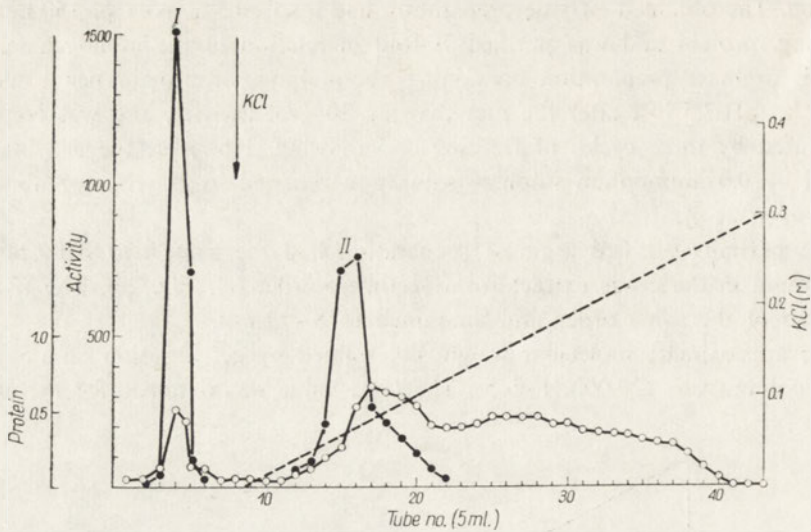rapidly than the second fraction.

Fig. 6. Chromatography of rat-liver arginase on DEAE-cellulose. The arginase preparation was obtained from rat liver as described for ox-brain arginase, and the dialysed 0.9 (NH₄)₂SO₄ sat. precipitate (5 ml., containing 30 mg. of protein) was applied to the column (1 × 20 cm.) and eluted as described in Fig. 4. (○), Protein (mg./ml.); (●), arginase activity (mμmoles of ornithine/min./ml. of eluate); (— — —), KCl concentration gradient.

When the arginase from rat liver isolated by the procedure employed for brain arginase was chromatographed on DEAE-cellulose, two active fractions were obtained (Fig. 6). The first, which accounted for more than a half of the recovered enzyme activity, was eluted with the buffer, and the second was eluted at about 0.06 mM-KCl concentration. The two liver isoenzymes did not differ in temperature stability and had the same molecular weight, 120 000, as determined by Sephadex G-100 gel filtration.

The isoenzymes of arginases from ox brain and rat liver were resolved by DEAE-cellulose chromatography in a similar manner. However, they differed in quantitative distribution and in that fraction *II* from the brain was more strongly adsorbed on the column than the appropriate fraction of liver arginase, and was eluted at a twice as high KCl concentration.

It should be noted that two isoenzymes of human-liver arginase resolved on CM-cellulose column, have been reported by Bascur, Cabello, Veliz & Gonzalez (1966), and probably two isoenzymes are present also in human erythrocytes (Cabello, Prajoux & Plaza, 1965).

Comparison of arginases from brain and liver

The activity of arginase found in ox brain was about 800 times lower in comparison with the activity in ox liver (Table 4).

Although in the brain no enzymes of the urea biosynthesis cycle, except argi-

Table 4

Arginase activity in homogenates from ox brain, ox liver and rat liver

The tissue was homogenized with 9 vol. of 0.05 M-$MnCl_2$ - 0.1 M-KCl - 0.01 M-tris-HCl buffer, pH 7.5. The homogenate was incubated with L-arginine as described in Methods. Protein was determined by the method of Lowry *et al.* (1951), after being precipitated with trichloroacetic acid and dissolved in 1 N-NaOH.

Tissue	Activity	
	$m\mu$moles/g. fresh tissue/min.	$m\mu$moles/mg. protein/min.
Rat liver	282 000	1660
Ox liver	87 000	1070
Ox brain	100	2.3

ninosuccinate lyase (Walker, 1958; Ratner *et al.*, 1960; Tomlinson & Westall, 1960), have been demonstrated, the brain arginase has the same properties as the enzyme found in the liver of ureotelic vertebrates.

By Sephadex-gel filtration the molecular weight of brain arginase was determined to be 120 000, and the same value was obtained for the rat-liver enzyme. The molecular weight of rat-liver arginase determined from sedimentation constant by Schimke (1962) and Mora *et al.* (1965) was 138 000, whereas for the liver "uricotelic" arginase it was 270 000 (Mora *et al.*, 1965).

The K_m value for brain arginase, similarly as for liver "ureotelic" enzyme, is about 9 mM, in contrast to liver "uricotelic" arginases (chicken and lizard) the K_m values of which are 100 - 200 mM (Mora *et al.*, 1965). The brain enzyme is inhibited by excess of substrate, a phenomenon which has been observed only with the "ureotelic" arginases.

Similarly as with liver arginases from ureotelic animals, two isoenzymes of brain arginase have been found to occur. So far, the physiological function of these isoenzymes is unknown.

REFERENCES

Andrews P. (1964). *Biochem. J.* **96**, 595.

Bach S. J. & Killip J. D. (1961). *Biochim. Biophys. Acta.* **47**, 336.

Bach S. J., Hawkins R. A. & Swaine D. (1963). *Biochem. J.* **89**, 263.

Bascur L., Cabello J., Veliz M. & Gonzalez A. (1966). *Biochim. Biophys. Acta* **128**, 149.

Buniatian H. Ch. & Davtian M. A. (1965). *J. Neurochem.* **13**, 743.

Cabello J., Prajoux V. & Plaza M. (1965). *Biochim. Biophys. Acta* **105**, 583.

Chinard F. P. (1952). *J. Biol. Chem.* **199**, 91.

Engel M. G. & Engel F. L. (1947). *J. Biol. Chem.* **167**, 535.

Greenberg D. M., Bagot A. E. & Roholt O. A. Jr. (1956). *Arch. Biochem. Biophys.* **62**, 446.

Hogeboom G. H. (1960). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol 1, p. 16. Acad. Press, New York.

Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. (1951). *J. Biol. Chem.* **193**, 265.

Mora J., Tarrab R., Martuscelli J. & Soberon G. (1965). *Biochem. J.* **96**, 588.

Ratner S., Morell H. & Caravalho E. (1960). *Arch. Biochem. Biophys.* **91**, 280.

Schimke R. T. (1962). *J. Biol. Chem.* **237**, 459.

Schütte R. (1943). *Hoppe Seyler's Z. physiol. Chem.* **279**, 52.

Sporn M. B., Dingman W., Defalco A. & Davies R. K. (1959a). *J. Neurochem.* **5**, 62.

Sporn M. B., Dingman W., Defalco A. & Davies R. K. (1959b). *Nature (Lond.)* **183**, 1520.

Tomlinson S. & Westall R. G. (1960). *Nature (Lond).* **188**, 235.

Walker J. B. (1958). *Proc. Soc. exp. Biol. (N.Y.)* **98**, 7.

Warburg O. & Christian W. (1941). *Biochem. Z.* **310**, 384.

Whittaker V. P., Michaelson I. A. & Kirkland R. J. A. (1964). *Biochem. J.* **90**, 293.

BADANIA NAD ARGINAZĄ Z MÓZGU WOŁU

Streszczenie

1. Wyizolowano arginazę z mózgu wołu i oczyszczono około 70-krotnie.

2. Enzym posiada następujące własności: dwa optima pH przy 7,5 oraz 9,5; K_m 9 mM; ciężar cząsteczkowy 120 000. Enzym jest aktywowany jonami Mn^{2+}, hamowany nadmiarem substratu oraz hamowany kompetytywnie przez ornitynę i lizynę.

3. Enzym jest zlokalizowany w jądrach i mitochondriach.

4. W mózgu wołu i w wątrobie szczura stwierdzono dwa izoenzymy arginazy; izoenzymy nie różniły się wartościami K_m ani ciężarem cząsteczkowym.

T. CHOJNACKI, A. PASZEWSKI and TERESA SAWICKA

THE FORMATION OF UDP-GLUCOSE AND UDP-GALACTOSE IN WILD TYPE AND MUTANTS OF *ASPERGILLUS NIDULANS*

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, Warszawa 12

1. The formation of UDPglucose and UDPgalactose in extracts of *Aspergillus nidulans* was studied with the aid of ^{32}P-labelled phosphosugars. 2. UTP : α-D-glucose--1-phosphate uridylyltransferase (EC 2.7.7.9) was separated from UDPG : α-D--galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) by gel filtration on Sephadex G-200. Their molecular weights were 62 000 and above 200 000, respectively. The K_m values of both enzymes were estimated. 3. The wild strain was able to produce 3 - 5 times as much of the UDPGal synthetizing enzyme when grown on lactose or galactose; a mutant lacking activity of this enzyme is described. No changes of the activity of UDPG synthetizing enzyme were found when lactose or galactose instead of glucose were used as a carbon source.

The studies on galactose utilization in *Aspergillus nidulans* performed by Roberts (1963, 1964) demonstrated that a metabolic pathway similar to that found in yeast, animals and bacteria was operative in this species. The utilization of galactose depends on the following reaction sequence (Kalckar, 1958):

$$\text{galactose} \dashrightarrow \text{Gal-1-P} \dashrightarrow \text{UDPGal} \dashrightarrow \text{UDPG}$$
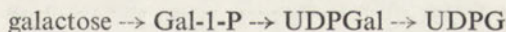
Though the induction in *Aspergillus nidulans* of enzymes of this pathway and genetic defects concerning individual enzymes have already been subjects of work carried out by Roberts (1963, 1964), the characteristics of the step in which Gal-1-P[1] reacts with the uridylyl donor was not studied in detail. The aim of the present paper was to study the UDPGal synthetizing enzyme (EC 2.7.7.12) in *Aspergillus nidulans* and to compare its properties with UDPG synthetizing enzyme (EC 2.7.7.9). The data on the activities of these two enzymes in the organism grown on various media and in some mutants are also reported.

---

[1] Abbreviations used: G-1-P, glucose-1-phosphate; Gal-1-P, galactose-1-phosphate; UDPG, uridine diphosphate glucose; UDPGal, uridine diphosphate galactose.

MATERIALS AND METHODS

Chemicals. ATP, CTP, GTP, ITP, TTP, UTP and UDPGal (sodium salts) were obtained from Sigma Chem. Co. (St. Louis, Mo., U.S.A.). UDPG was the product of Nutr. Biochem. Corp. (Cleveland, Ohio, U.S.A). Charcoal from Zakł. Elektr. Węgl. (Racibórz, Poland) was treated before use according to Threlfall (1957). Sephadex G-200 was from Pharmacia (Uppsala, Sweden). Protein standards for molecular weight estimations were: human albumin, human γ-globulin, products of Warsaw Serum and Vaccine Plant (Poland), and egg white lysozyme from Reanal (Budapest, Hungary). [^{32}P]Galactose-1-phosphate and [^{32}P]glucose-1--phosphate (sp. activity 5×10^5 to 4×10^6 counts/min./µmole) were prepared as described in the previous paper (Sawicka & Chojnacki, 1969).

Biological material. Wild type strain of *Aspergillus nidulans* was obtained from the Department of Genetics, University of Glasgow (Great Britain). Two mutants *lac 19* and *lac 24* slowly growing on lactose were isolated in our Institute by Mrs. Jadwiga Litwińska.

Culture conditions and preparation of enzyme extracts. Mycelium was grown in 250 ml. Erlenmeyer flasks containing 100 ml. of minimal medium (Cove, 1966) with glucose, galactose, lactose or sodium acetate as carbon source. Flasks were inoculated with 2 - 3 ml. of a heavy conidial suspension in water and incubated at 32° for 30 - 40 hr. on a rotatory shaker. Mycelium was then filtered off on cheese--cloth and washed with distilled water. Blotted mycelial pads were mixed with glass powder and ground in a chilled mortar with 7 - 8 volumes of 0.1 м-sodium phosphate buffer, pH 7.4. The resulting slurry was centrifuged at 15 000 g for 15 min. at 4° and the supernatant was used for experiments.

Enzyme assays. For the assay of UDPGal synthetizing enzyme the reaction mixture contained in a final volume of 0.4 ml.: [^{32}P]Gal-1-P, 0.05 µmole; UDPG, 0.1 µmole; EDTA, 1.2 µmoles; glycine buffer, pH 8.1, 20 µmoles, and 0.01 - 0.05 ml. of mycelial extract or 0.1 ml. of the effluent from Sephadex G-200 column.

For the assay of UDPG synthetizing enzyme the reaction mixture contained in a volume of 0.4 ml.: [^{32}P]G-1-P, 0.1 µmole; UTP, 0.1 µmole; MgCl$_2$, 6 µmoles; tris-HCl buffer, pH 7.5, 10 µmoles; and 0.01 - 0.05 ml. of mycelial extract or 0.1 ml. of the effluent from Sephadex G-200 column.

The reaction mixtures were incubated for 15 min. at 37°. In both assays the reaction was stopped by immersing the tubes in ice and adding 0.4 ml. of 10% trichloroacetic acid. The amount of radioactive nucleotide formed was estimated as described previously (Chojnacki, Sawicka & Korzybski, 1968).

Other methods. Gel filtration of the mycelial extracts was performed as described previously (Chojnacki *et al.*, 1968). Ion exchange paper chromatography (descending) was performed using polyethyleneimine-impregnated Whatman no. 1 paper and 0.2 м-LiCl as solvent (Verachtert, Bass, Wilder & Hansen, 1966). The methods of estimation of ^{32}P, phosphorus and protein were the same as used previously (Chojnacki *et al.*, 1968).

RESULTS

The results presented in Table 1 demonstrate that only uridine nucleotides can activate the studied hexose phosphates. G-1-P reacted with UTP and slightly with UDPG, and Gal-1-P with UDPG. In this experiment relatively large amounts of mycelial protein were added in order to visualize any enzymic activity of the extract. In further experiments on quantitative estimation of the activity of UDPG synthetizing enzyme and UDPGal synthetizing enzyme the amounts of mycelial protein used for the tests were at least 20 times lower. In these conditions during 15 min. incubation no detectable decomposition of the substrates and products of the studied reaction was observed, and up to 30 min. the reaction was linear with time. Substrate affinities of UDPG synthetizing enzyme for UTP and G-1-P determined in diluted crude mycelial extract were 0.2 mm and 0.19 mm, respectively, the values of UDPGal synthetizing enzyme for UDPG and Gal-1-P were 0.22 mm and 0.1 mm, respectively.

The activities of both enzymes in mycelia grown on various media are presented in Table 2. In the organism grown on lactose or galactose a 3 - 5-fold increase of activity of UDPGal synthetizing enzyme was observed as compared with mycelia grown on glucose or acetate. The activity of UDPG synthetizing enzyme was the same in mycelia grown on galactose, lactose, glucose and acetate. The results presented in Table 2 are taken from two series of cultures where the activities of the studied enzymes in mycelia grown on all the four types of media were assayed in the same experiment. The occurrence of induction of UDPGal synthetizing enzyme by lactose and galactose is well visible in both series. The absolute values of enzyme activities varied from one culture to another, but the induction was observed in all performed experiments.

In Table 3 are presented the results of the assays of activity of both enzymes in two mutants and in wild type of *A. nidulans*. The mutants *lac 19* and *lac 24* both grow on lactose a few times slower than the wild type; *lac 19* does not grow on galactose and *lac 24* grows on this hexose slower than on glucose. The assays show a lack of UDPGal synthetizing enzyme in *lac 19*. The rate of UDPG formation in this mutant was comparable both with that in *lac 24* and in the wild type. *Lac 24* does not differ from the wild type with respect to these two enzymes.

The incorporation of Gal-1-P into nucleotide material was checked also by means of ion exchange paper chromatography. It can be seen on the autoradiogram of the chromatogram (Fig. 1) that ^{32}P was incorporated from Gal-1-P by mycelial extract mainly into compounds of R_F similar to that of UDPG and UDPGal. These two nucleotides could not be separated by this chromatographic system. In addition, formation of an unidentified radioactive compound with a high R_F value was noted. Only a small part of the substrate was decomposed to orthophosphate upon incubation with mycelial extract.

The two enzymes responsible for the activation of G-1-P and Gal-1-P were separated by gel filtration on Sephadex G-200. A typical elution pattern is pre-

Table 1

Formation of nucleoside diphosphate sugars from [^{32}P]*glucose-1-phosphate and* [^{32}P]*ga-lactose-1-phosphate in extracts of wild type strain of* Aspergillus nidulans

The reaction mixtures were as described in Material and Methods except that UTP or UDPG were omitted or replaced by other nucleoside-5′-triphosphates (0.1 μmole) as indicated. Each sample contained 10 μl. of crude extract of *Aspergillus nidulans* (0.03 mg. of protein) and was incubated for 15 min. at 37°.

Addition	G-1-P	Gal-1-P
	mμmoles incorporated into nucleotides per sample	
None	0.1	< 0.1
ATP	0.1	< 0.1
CTP	0.2	< 0.1
GTP	0.1	< 0.1
ITP	0.0	< 0.1
TTP	0.4	< 0.1
UDPG	2.9	26.0
UTP	16.8	< 0.1

Table 2

The incorporation of [^{32}P]*G-1-P and* [^{32}P]*Gal-1-P into nucleotides in extracts of the wild type of* Aspergillus nidulans *grown on various media*

The incorporation of ^{32}P into UDPG or UDPGal is expressed in mμmoles of radioactive nucleotide per mg. protein per minute.

Substrate	Expt. no.	Carbon source in the culture			
		acetate	glucose	lactose	galactose
[^{32}P]G-1-P	a	54.6	72.0	44.6	56.6
	b	221.0	270.6	278.0	237.0
[^{32}P]Gal-1-P	a	12.3	11.3	29.3	28.0
	b	18.2	18.6	100.0	66.8

Table 3

The incorporation of [^{32}P]*G-1-P and* [^{32}P]*Gal-1-P into nucleotides in extracts of three strains of* Aspergillus nidulans *grown on glucose*

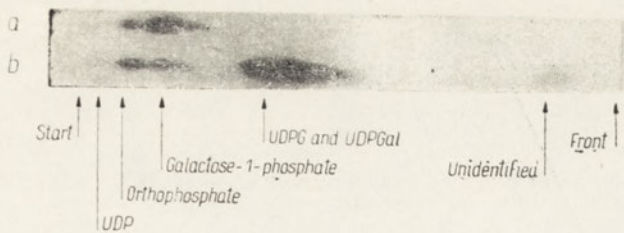| Extract from	G-1-P	Gal-1-P
	mμmoles incorporated into nucleotides per mg. protein per minute	
Wild type	130	10.7
lac 24	168	10.4
lac 19	280	0.0

Fig. 1. Autoradiogram of paper chromatogram of (*a*) [^{32}P]Gal-1-P and (*b*) of the products formed from [^{32}P]Gal-1-P and UDPG in the extract of the wild type of *Aspergillus nidulans*. For this experiment an amount of extract corresponding to 0.5 mg. of protein was used and incubation was carried out for 30 min. A sample of the mixture was spotted directly on polyethyleneimine-impregnated Whatman no. 1 paper and developed with 0.2 M-LiCl.
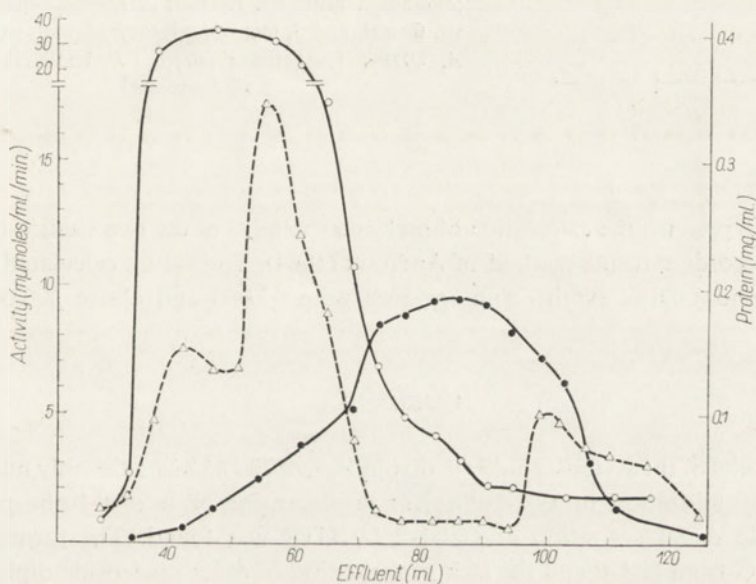

Fig. 2. Separation of UDPG synthetizing enzyme from UDPGal synthetizing enzyme from wild type strain of *Aspergillus nidulans* by gel filtration on Sephadex G-200. The 15 000 *g* supernatant of mycelial homogenate (2 ml.) was applied to a 1.5 × 63 cm. column of Sephadex G-200 and eluted with 0.145 M-NaCl - 0.005 M-tris-HCl buffer, pH 7.5. Fractions of 2 ml. were collected. (○), UDPG synthetizing enzyme; (●), UDPGal synthetizing enzyme; both activities expressed in mμmoles of radioactive uridine diphosphate sugars formed per 1 ml. per minute; (△), amount of protein (mg./ml.).

sented in Fig. 2. The UDPG synthetizing enzyme emerged first from the column together with the bulk of mycelial proteins and was followed by the smaller peak of UDPGal synthetizing enzyme.
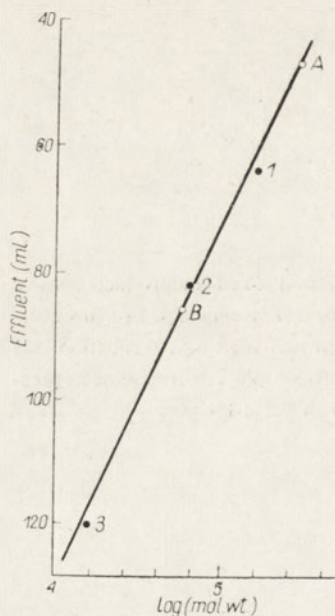
http://rcin.org.pl

Fig. 3. Determination of molecular weights of UDPG and UDPGal synthetizing enzymes of wild strain of *Aspergillus nidulans* by Sephadex G-200 gel filtration. In the eluate from a 1.5×63 cm. column the elution volumes of the enzymes studied were compared with those of proteins of known molecular weight as described by Andrews (1964). Standard proteins: *1*, human γ-globulin, mol. wt. 150 000; *2*, human albumin, mol. wt. 69 000, and *3*, egg white lysozyme, mol. wt. 17 500; *A*, UDPG synthetizing enzyme; *B*, UDPGal synthetizing enzyme.

Figure 3 presents the calculation of molecular weights of the two studied mycelial enzymes according to the method of Andrews (1964). The values calculated for the UDPGal and UDPG synthetizing enzymes were 62 000 and above 200 000, respectively.

DISCUSSION

The results of these studies indicate that in *Aspergillus nidulans* the only nucleotide pathway of utilization of Gal-1-P is *via* displacement of hexose-1-phosphate in UDPG. No direct activation of Gal-1-P by UTP was found. The formation of only UDPG from G-1-P and the lack of formation of other nucleoside diphosphate glucose when UTP was replaced by other nucleoside-5'-triphosphate indicate that rather simple types of transglycosylation reactions occur in this organism that could be mediated only by the uridine coenzyme. It should be mentioned that a variety of well separable pyrophosphorylases catalyse the reaction of G-1-P with various nucleoside-5'-triphosphates in *S. typhimurium* (Chojnacki *et al.*, 1968) where more complex transglycosylations lead to the formation of specific polysaccharides.

The elution pattern of the two *Aspergillus nidulans* enzymes from Sephadex closely resembled that observed for enzymes of human erythrocytes (Sawicka & Chojnacki, 1969). There was no cross unspecificity of the enzymes towards the substrates.

On the basis of Robert's paper (1964), the mutant *lac 19* can be placed in locus *gal-5* located in the left arm of the I chromosome of *Aspergillus nidulans* (Roberts, 1963). This locus is probably a structural gene for UDPGal synthetizing enzyme.

The 3 - 5-fold increase of the activity of this enzyme upon induction by galactose or lactose observed in this work agrees with that reported by Roberts (1964). It is very likely that galactose or galactose-1-phosphate acts as an inducer in the system controlling the rate of synthesis of this enzyme. Glucose evidently does not interfere with the regulation as equal induction was observed in mycelia grown on galactose and on lactose. Roberts (1964) found a gene responsible for this regulation. It is the *gal-1* locus situated in the left arm of the III chromosome. It should be noted that the same gene controls the synthesis of galactokinase (EC 2.7.1.6) but not of UDPglucose epimerase (EC 5.1.3.2). It is clear from the described experiments that the UDPG synthetizing enzyme must be controlled by a regulation system different from that controlling the UDPGal synthetizing enzyme.

REFERENCES

Andrews P. (1964). *Biochem. J.* **91**, 222.
Chojnacki T., Sawicka T. & Korzybski T. (1968). *Acta Biochim. Polon.* **15**, 293.
Cove D. J. (1966). *Biochim. Biophys. Acta* **113**, 51.
Kalckar H. (1958). *Advanc. Enzymol.* **20**, 111.
Roberts C. F. (1963). *J. Gen. Microbiol.* **31**, 45.
Roberts C. F. (1964). *Genetics* **50**, 279.
Sawicka T. & Chojnacki T. (1969). *Clin. Chim. Acta* **23**, 463.
Threlfall C. J. (1957). *Biochem. J.* **65**, 694.
Verachtert H., Bass S. T., Wilder J. K. & Hansen R. G. (1966). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.), vol. 8, p. 111. Academic Press, New York, London.

WYTWARZANIE UDP-GLUKOZY I UDP-GALAKTOZY W SZCZEPIE DZIKIM I MUTANTACH GRZYBA *ASPERGILLUS NIDULANS*

Streszczenie

1. Badano tworzenie UDPglukozy i UDPgalaktozy w ekstraktach ze szczepu dzikiego i dwóch mutantów *Aspergillus nidulans* stosując znakowane ^{32}P glukozo-1-fosforan i galaktozo-1-fosforan.
2. Za pomocą filtracji na żelu Sephadex G-200 oddzielono enzym syntetyzujący UDPglukozę (EC 2.7.7.9) od enzymu syntetyzującego UDPgalaktozę (EC 2.7.7.12) i określono masy cząsteczkowe tych enzymów.
3. Stwierdzono 3 - 5-krotne zwiększenie aktywności enzymu syntetyzującego UDPgalaktozę jeśli grzybnię hodowano na laktozie lub galaktozie zamiast na glukozie. Drugi z badanych enzymów nie ulegał indukcji.
4. Opisano mutanta pozbawionego aktywności enzymu syntetyzującego UDPgalaktozę.

B. GORZKOWSKI

UTILIZATION OF ^{14}C-LABELLED PURINE PRECURSORS FOR URIC ACID SYNTHESIS IN *HELIX POMATIA*

Department of Physiological Chemistry, Medical School, Krakowskie Przedmieście 26/28, Warszawa 64

1. In the uricotelic land snail, *Helix pomatia*, the incorporation *in vivo* of [^{14}C]formate, [1-^{14}C]glycine and $NaH^{14}CO_3$ into excreted uric acid, guanine and xanthine was demonstrated. 2. The radioactive uric acid isolated from nephridia was submitted to chemical degradation to determine the label of individual carbon atoms. 3. It was found that in *Helix pomatia*, similarly as in birds, carbons 4 and 5 of uric acid are derived from glycine, carbons 2 and 8 from formate, and carbon 6 from bicarbonate.

It has been found in this laboratory that, contrary to earlier views, the snail *Helix pomatia* is an uricotelic animal during the whole year (Jeżewska, Gorzkowski & Heller, 1963a,b). Over 90% of the excreted nitrogen was identified as uric acid, xanthine and guanine. During hibernation the snails stored the purines in nephridium, and at the end of hibernation the amount of purines was as high as 750 μmoles. Also during the feeding period the nephridium contained rather large amounts of purines.

Uricotelism occurs in invertebrate animals adapted to life on land. In invertebrates, the type of nitrogen excretion is not dependent on the phylogenetic development of the animal but on environmental conditions. The pathway of purine biosynthesis in vertebrates is well established; the first purine compound is inosinic acid from which adenylic acid and guanilic acid are formed. In uricotelic animals, the biosynthesis of excreted uric acid could be expected to proceed in the same way.

In our experiments with [^{14}C]glycine (Jeżewska, Gorzkowski & Heller, 1964) it has been demonstrated that the snail *Helix pomatia* incorporates this compound to a large extent into purines. Radioactivity of uric acid was located mainly in carbons 4 and 5.

In the present work, three precursors of purine synthesis in vertebrates, [^{14}C]formate, [1-^{14}C]glycine and $NaH^{14}CO_3$, were administered to *Helix pomatia* and it has been found that there are no differences in the position of their incorporation in the snail and in birds. A preliminary account of this work has been presented (Gorzkowski, 1965).

Lee & Campbell (1965) studied the synthesis of uric acid in another land snail, *Otala lactea*, using ^{14}C-labelled precursors of inosinic acid and demonstrated the incorporation of the label into uric acid.

MATERIALS AND METHODS

Reagents. Sodium [14]Cbicarbonate (0.97 mc/m-mole) and [1-14]Cglycine (0.92 mc/m-mole) were products of the Radiochemical Centre (Amersham, England). Sodium [14]Cformate (0.104 mc/m-mole) was a U.S.S.R. product. 2,5-Diphenyloxazole was from Nuclear Enterprises (Edinburgh, Great Britain). Dowex 50 X4, 100 - 200 mesh, was from Fluka A.G. (Buchs, Switzerland). Other reagents were RA grade (Fabryka Odczynników Chemicznych, Gliwice, Poland). Naphthalene was sublimated just before use; methanol and ethyl glycol were dehydrated and distilled.

Measurement of radioactivity. A scintillation counter (S.E.2, Poland) was used. The efficiency of counting amounted to 30%.

The scintillation medium was prepared according to Bray (1960) and contained: naphthalene, 0.6 g.; 2,5-diphenyloxazole, 0.04 g.; methanol, 1.0 ml.; ethylene glycol, 2.0 ml.; and dioxane to a volume of 10 ml.

Isolation of purines. The purines were separated by column chromatography on Dowex 50 X4 (H$^+$ form) 100 - 200 mesh, as described in the text. In the eluates, the purines were identified by U.V. absorption spectra and by the characteristic E_{250}/E_{260} and E_{280}/E_{260} ratios at pH 2 and 9, 10 or 11. Concentration of purines in the eluate was calculated from molar extinctions at pH 2 (Volkin & Cohn, 1959).

Animals. The snails *Helix pomatia* were collected in autumn, by the end of the feeding period, and used for experiments within a week; durig this period they were fed lettuce leaves.

RESULTS

Incorporation of the labelled precursors into purines in vivo

Purine precursors, sodium [14]Cbicarbonate, sodium [14]Cformate and [1-14]Cglycine were injected into the hepatopancreas of *Helix pomatia* during the feeding period. The animals were kept in a tightly covered container, and three days after the injection, nephridia were isolated and the crystalline deposit of purines was washed out with 10 ml. of water. The suspension of nephridial content was centrifuged for 10 min. at 8000 rev./min. The heavy purine crystals sedimented on the bottom of the tube, and over them a layer of light nephridial debris was formed. The supernatant fluid and the tissue debris layer were decanted and heated to boiling. The precipitated proteins were discarded by centrifugation and the supernatant, containing the purines which had remained in the debris, was added to the main sediment of purines. Then Li_2CO_3 *in substantia* was added, and warm water was poured in until all purines were dissolved; about 300 - 500 ml. of water was used
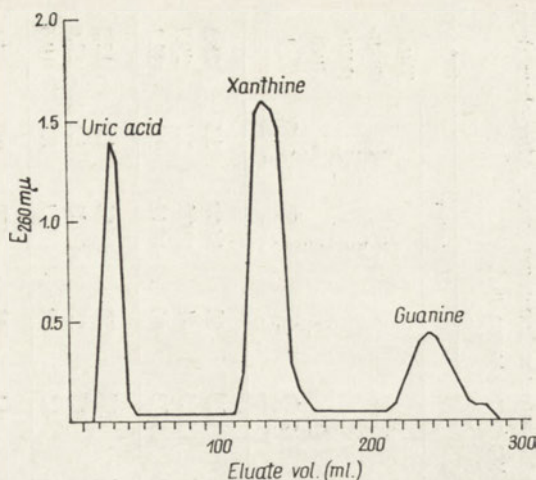
Fig. 1. Chromatogram of purines isolated from nephridium, on a column (1×20 cm.) of Dowex 50X4 (H+ form, 100 - 200 mesh). Purines were eluted with a HCl concentration gradient up to 2 N. The mixing vessel contained 50 ml. of water and the reservoir 100 ml. of 0.7 N-HCl and then 200 ml. of 2.0 N-HCl. Fractions of 5 ml. were collected.

to solubilize the content of one nephridium. The hot solution was cleared by filtration. To separate the purines, 10 ml. of the solution was applied to a column of Dowex 50 X4 and eluted with a HCl concentration gradient up to 2 N (Fig. 1). From the molar extinction of the appropriate purines at pH 2, their concentrations in the fraction were calculated and radioactivity was measured in a sample containing not more than 0.1 mg. of purine. The sample was evaporated to dryness under vacuum to remove HCl, the residue was dissolved in 0.2 N-NaOH, and radioactivity was determined in the scintillation medium.

All three precursors were incorporated into purines. Three days after the injection, formate was incorporated to the greatest extent, 29 - 32% of the radioactivity, glycine in 11 - 12%, and bicarbonate only in 2% (Table 1). The radioactivity from each of the three precursors was recovered mainly in uric acid and xanthine, a smaller amount being found in guanine.

Distribution of radioactivity in uric acid carbons

To obtain a greater amount of purified uric acid, the solution of purines from one nephridium was added with acetic acid until a sediment appeared, and left overnight in a refrigerator. The sediment containing uric acid, guanine, and some undissolved xanthine was centrifuged, washed with cold water, and the remaining traces of acetic acid were evaporated under reduced pressure. To the residue, hot water was added, the undissolved guanine and a part of uric acid were discarded by centrifugation, and the supernatant solution was applied to the Dowex 50 X4 (H+ form) column. Uric acid was eluted with 0.3 N-HCl; at this HCl concentration,

Table 1

Incorporation in vivo of ^{14}C-labelled precursors into excreted purines in the snail

Sodium [^{14}C]formate (4 μc), [1-^{14}C]glycine (6 μc) or NaH^{14}CO$_3$ (6 μc, and after 24 hr. 4 μc) was injected into the hepatopancreas. After three days, nephridia were isolated, purines separated, and their amounts and radioactivities were determined.

Compound injected	Activity administered (counts/min.)	Expt. no.	Uric acid			Xanthine			Guanine			Total incorporation (%)
			μmoles	counts/μmole/min.	Incorporation (%)	μmoles	counts/μmole/min.	Incorporation (%)	μmoles	counts/μmole/min.	Incorporation (%)	
Sodium [^{14}C]formate	2.49·10^6	1	136	2 381	13.0	127	2 411	12.3	52	1 660	3.5	28.8
		2	142	2 610	14.9	166	1 755	11.7	41	1 902	3.1	29.7
		3	160	2 428	15.6	178	1 860	13.3	46	1 712	3.2	32.1
[1-^{14}C]glycine	3.87·10^6	4	125	1 773	5.7	131	1 544	5.2	38	1 462	1.4	12.3
		5	153	1 482	5.9	164	1 121	4.7	55	1 023	1.4	12.0
		6	118	1 593	4.9	136	1 283	4.5	40	1 119	1.2	10.6
NaH^{14}CO$_3$	6.71·10^6	7	107	714	1.1	128	441	0.8	44	360	0.2	2.1
		8	124	651	1.2	142	485	1.0	61	317	0.3	2.5
		9	97	646	0.9	131	395	0.8	46	328	0.2	1.9

uric acid was completely eluted whereas xanthine emerged at a HCl concentration exceeding 1 N, and guanine at a still higher concentration.

The obtained samples of uric acid which were spectrophotometrically pure were recrystallized and submitted to degradation to determine the radioactivity of the individual carbons (the scheme of degradation is shown in Fig. 2).
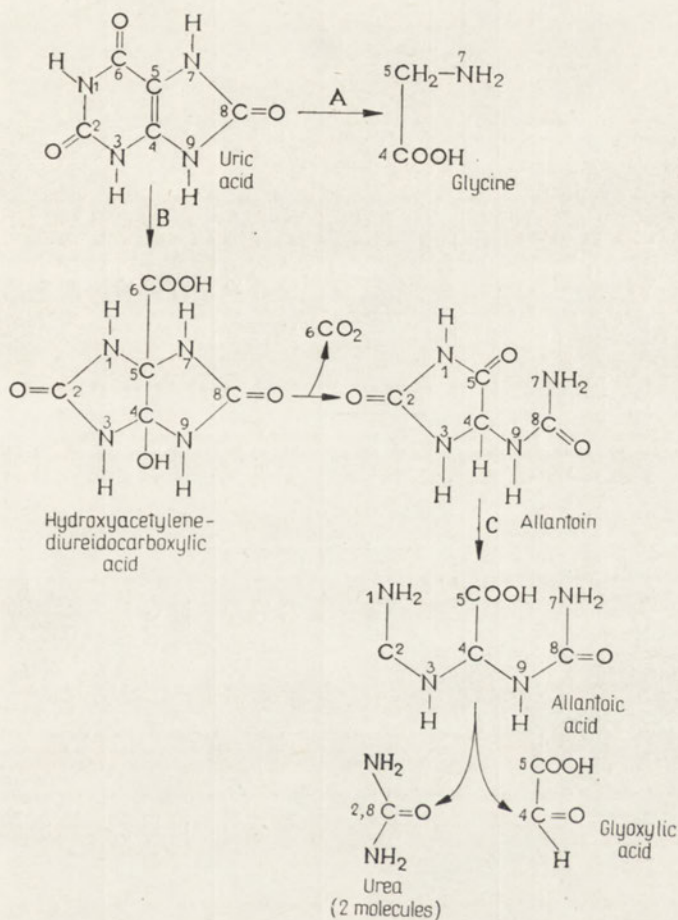

Fig. 2. Scheme of degradation of uric acid: A, according to the method of Wulff (Korn, 1957); B and C, according to the method of Buchanan *et al.* (1948).

The radioactivity of carbons 4 and 5 was determined together. Uric acid was degraded by the method of Wulff as described by Korn (1957). In a sealed tube, 6 mg. of uric acid and 0.5 ml. of concentrated HCl were heated at 160° for 18 hr. Under these conditions, glycine is formed from carbons 4 and 5 and nitrogen 7. Glycine was isolated by ascending chromatography on Whatman no. 4 paper using water as solvent (Burma & Banerjee, 1950). After localization of glycine on a narrow

Table 2

Distribution of radioactivity in individual carbons of uric acid

Urid acid, obtained from snail nephridium after injection into the hepatopancreas of ^{14}C-labelled compounds in the amounts given in Table 1, was degraded and the radioactivity of the obtained products determined. For details see text. From the specific radioactivity of uric acid and specific radioactivities of glycine, urea and CO_2, the percentages of radioactivity of uric acid found in the degradation products were calculated.

Compound injected	Expt. no.	Uric acid spec. act. (counts/μmole/min.)	Radioactivity of					
			C-4 and C-5 (glycine)		C-2 and C-8 (urea)		C-6 (CO_2)	
			counts/μmole/min.	% of uric acid activity	counts/μmole/min.	% of uric acid activity	counts/μmole/min.	% of uric acid activity
Sodium [^{14}C]formate	1	2 381	35	1.4	1 173	98.5	0	0
	2	2 610	55	2.1	1 283	98.3	0	0
	3	2 428	43	1.7	1 176	96.9	0	0
[1-^{14}C]Glycine	4	1 773	1 635	92.2	25	2.8	87	5.3
	5	1 482	1 268	85.6	23	3.1	90	6.1
	6	1 593	1 413	88.7	26	3.1	65	4.3
NaH^{14}CO$_3$	7	714	177	24.8	0	0	508	71.4
	8	651	152	23.3	0	0	495	76.0
	9	646	145	26.2	0	0	448	69.4

strip of the chromatogram, the appropriate area was eluted with water, the eluate was concentrated by evaporation and rechromatographed in a solvent system of *n*-propanol - water (7:3, v/v) according to Opieńska-Blauth, Kowalska & Pietrusiewicz (1956). The amount of glycine was determined by the method of Jacobs (1956), and radioactivity with the scintillation counter.

Degradation of uric acid to allantoin and CO_2, which represents carbon 6, was carried out according to Buchanan, Sonne & Delluva (1948) by oxidation with MnO_2. The evolved CO_2 was collected in NaOH solution and its specific radioactivity was determined.

Radioactivity of carbons 2 and 8 was determined together by the method of Buchanan *et al.* (1948). The allantoin obtained in the previous procedure was converted by heating in alkaline medium into allantoic acid, which was then decomposed into two molecules of urea and glyoxalic acid. Urea was not further degraded as in the method of Buchanan but isolated by ascending chromatography on Whatman no. 3 paper in a solvent system of *n*-butanol - acetic acid - water (4:1:5, by vol.) according to Noworytko & Sarnecka-Keller (1956). Urea was located with a 1% solution of *p*-dimethylaminobenzaldehyde in 15% HCl, and the appropriate area eluted with water. The concentration of urea was measured by the xanthydrol method of Engel & Engel (1947), and its specific radioactivity determined.

The radioactivity of uric acid and its distribution in the individual carbons after administration of [^{14}C]formate, [1-^{14}C]glycine and $NaH^{14}CO_3$ is presented in Table 2. In experiments with formate, almost all the radioactivity of uric acid was found in carbons 2 and 8 (98%). Glycine was incorporated into carbons 4 and 5 (89%). Bicarbonate was incorporated mainly into carbon 6 (72%), the remaining activity being found in carbons 4 and 5.

The comparison of distribution of radioactivity in uric acid carbons in the snail with the values obtained by Karlsson & Barker (1949) in their studies on the pigeon, presented in Table 3, shows that the origin of uric acid carbons in the snail is the same as in vertebrates. Both the phylogenetically young birds, and the phylogenetically old snails, make use of the same path of purine biosynthesis.

Table 3

Comparison of ^{14}C-labelling of uric acid in the snail with the data obtained by Karlsson & Barker (1949) for the pigeon

The activities of individual carbons are given in percentages of the activity of uric acid submitted to degradation. The values for the snail are means from 3 experiments (Table 2).

Compound injected	Percentage of radioactivity in					
	C-2 and C-8		C-4 and C-5		C-6	
	snail	pigeon	snail	pigeon	snail	pigeon
Sodium [^{14}C]formate	97.9	98.5	1.7	1.3	0	0.2
[1-^{14}C]Glycine	3.0	0	88.8	97.6	5.2	2.8
$NaH^{14}CO_3$	0	0.7	24.8	23.2	72.3	75.0

REFERENCES

Bray G. A. (1960). *Analyt. Biochem.* **1**, 279.

Buchanan J. M., Sonne J. C. & Delluva A. M. (1948). *J. Biol. Chem.* **173**, 81.

Burma D. P. & Banerjee B. (1950). *Science and Culture (India)* **15**, 363.

Engel M. G. & Engel F. L. (1947). *J. Biol. Chem.* **167**, 535.

Gorzkowski B. (1965). *IV Symposium Polish Biochem. Soc., 11 - 12 June 1965, Abstr. Commun.*
    p. 63.

Jacobs S. (1956). *XV Congresso Internationale de Quimica Pura e Aplicada. Actas de Congresse*
    vol. 3, VIII-20, Lisboa.

Jeżewska M. M., Gorzkowski B. & Heller J. (1963a). *Acta Biochim. Polon.* **10**, 55.

Jeżewska M. M., Gorzkowski B. & Heller J. (1963b). *Acta Biochim. Polon.* **10**, 309.

Jeżewska M. M., Gorzkowski B. & Heller J. (1964). *Acta Biochim. Polon.* **11**, 135.

Karlsson J. L. & Barker H. A. (1949). *J. Biol. Chem.* **177**, 597.

Korn E. D. (1957). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 4, p. 615.
    Academic Press, New York.

Lee T. W. & Campbell J. W. (1965). *Comp. Biochem. Physiol.* **15**, 457.

Noworytko J. & Sarnecka-Keller M. (1956). *Acta Biochim. Polon.* **3**, 309.

Opieńska-Blauth J., Kowalska H. & Pietrusiewicz M. (1956), *Acta Biochim. Polon.* **3**, 557.

Volkin E. & Cohn W. E. (1959). In *Methods of Biochemical Analysis* (D. Glick, ed.) vol. 1, p. 304
    (Appendix). Interscience Publ., New York.

WYKORZYSTANIE PREKURSORÓW PURYN ZNAKOWANYCH ^{14}C
DO SYNTEZY KWASU MOCZOWEGO U *HELIX POMATIA*

Streszczenie

1. Wykazano wbudowywanie [^{14}C]mrówczanu, [1-^{14}C]glicyny i NaH^{14}CO$_3$ *in vivo* do wydalanych puryn: kwasu moczowego, gwaniny i ksantyny u urikotelicznego ślimaka lądowego *Helix pomatia*.

2. Radioaktywny kwas moczowy izolowany z nephridium poddawano chemicznej degradacji w celu ustalenia radioaktywności węgli w poszczególnych pozycjach pierścienia purynowego.

3. Stwierdzono, że u *Helix pomatia*, podobnie jak u ptaków, węgle 4 i 5 kwasu moczowego pochodzą z glicyny, węgle 2 i 8 z mrówczanu i węgiel 6 z dwuwęglanu.

T. KULIKOWSKI, BARBARA ŻMUDZKA and D. SHUGAR

PREPARATION AND PROPERTIES OF N^4-ALKYL ANALOGUES OF 5-METHYL-2'-DEOXYCYTIDINE, THEIR 5'-MONO- AND TRIPHOSPHATES, AND N^4-ALKYL DERIVATIVES OF 1,5-DIMETHYLCYTOSINE

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, Warszawa 12; and Department of Radiobiology, Institute of Oncology, ul. Wawelska 15, Warszawa 22

1. The synthesis and properties are reported of the N^4-mono methyl and ethyl amino analogues of 5-methyl-2'-deoxycytidine and 1,5-dimethylcytosine, and the N^4,N^4-dimethylamino analogue of 5-methyl-2'-deoxycytidine. 2. Particular attention was devoted to the problems associated with the preparation of N^4,N^4-dimethylamino analogues by the action of dimethylamine on 1-methyl-4-O-ethylthymine, 1-methyl-4-thiothymine and 3',5'-di-O-benzoyl-4-thiothymidine. 3. The ultraviolet absorption spectra and pK_a values of the various 1-substituted-N^4-alkylcytosines are described and discussed. 4. The syntheses of 5'-monophosphates and 5'-triphosphates of 5-methyl-2'-deoxycytidine and its alkylamino analogues have also been described. The triphosphates were elsewhere shown to be substrates of terminal deoxynucleotidyl transferase.

The purpose of the present investigation was the preparation of homopolydeoxynucleotides in which the base residues are 5-methylcytosine or its N^4-alkyl (methyl, ethyl) analogues. This required the preparation of the 5'-triphosphates of 5-methyl-2'-deoxycytidine and the N^4-alkylamino analogues of the latter, on the assumption that these would prove to be substrates of DNA polymerase and/or terminal deoxynucleotidyl transferase. It did, in fact, prove possible to prepare the foregoing polymers by enzymic methods, and the details of these preparations, as well as the properties of the polydeoxynucleotides obtained, are presented elsewhere (Żmudzka, Bollum & Shugar, 1968; Żmudzka, Bollum & Shugar, in press). This communication describes the preparation of the substrates and a number of structural analogues (Scheme 1), several of which have not hitherto been reported. Particular interest attaches to these substrates, their structural analogues, and the resulting polymers because of the widespread occurrence of 5-methylcytosine as a minor or major component in natural nucleic acids. Indeed, a bacteriophage has been recently isolated, the DNA of which contains exclusively 5-methylcytosine in place of cytosine (Kuo, Huang & Tang, 1968). Furthermore the availability of poly-5-methyl-2'--deoxycytidylic acid has made it possible to examine the influence of a 5-methyl
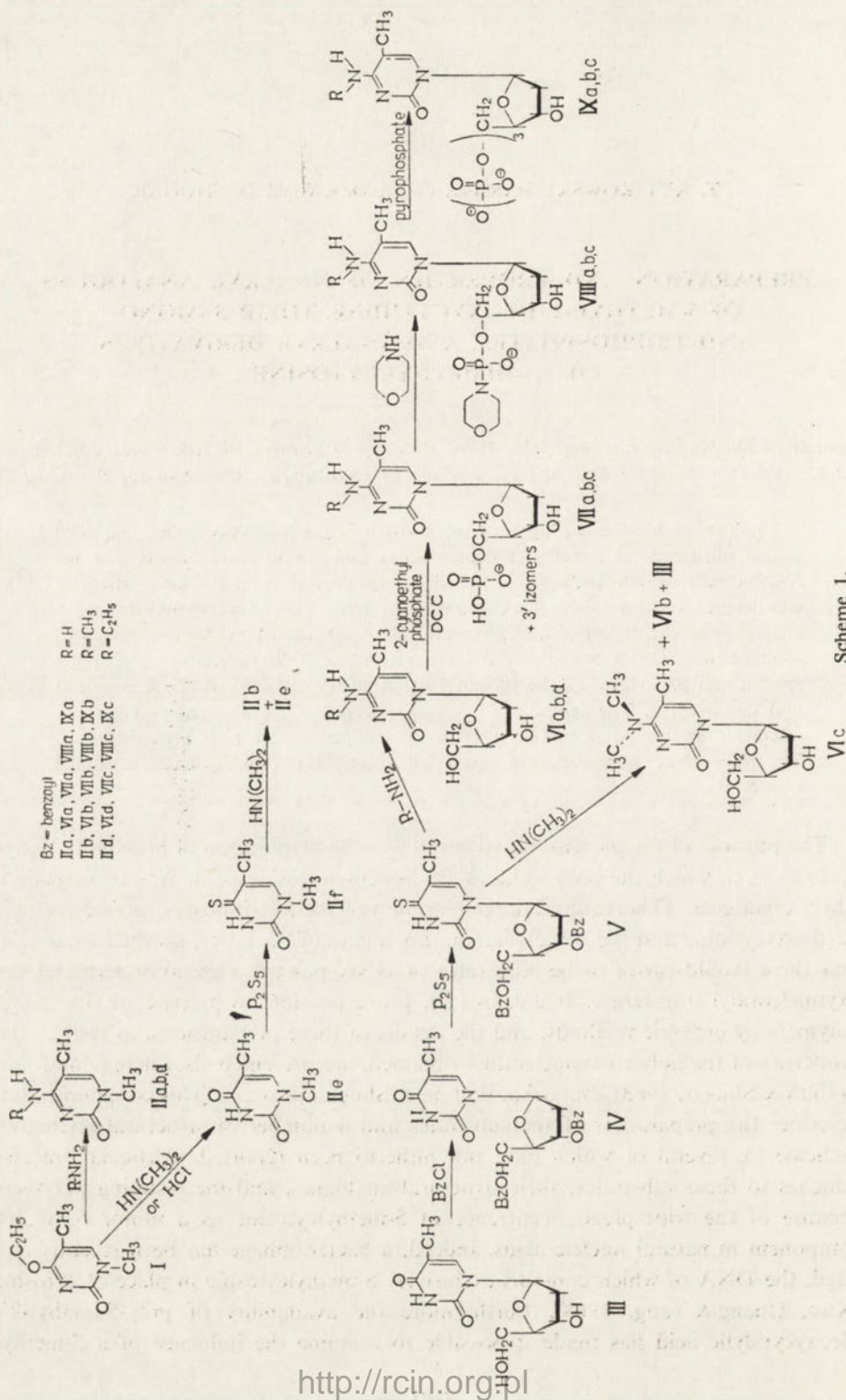
Scheme 1.

pyrimidine substituent on the properties of the acid twin-helical form of poly-de-oxycytidylic acid, as had already been achieved for the corresponding ribopolymers (Szer & Shugar, 1966b).

RESULTS AND DISCUSSION

Synthesis of deoxynucleosides

5-Methyl-2'-deoxycytidine (VIa) has previously been obtained *via* the thiation of thymidine (Fox *et al.*, 1959; Wempen, Ueda & Fox, 1963) and by direct condensation *via* the Hilbert-Johnson rearrangement (Prystaš, Farkaš & Šorm, 1965). In view of the ready availability of thymidine, the former of these was adopted, the more so in that it gives only the natural, β-anomer. Thiation of 3',5'-di-*O*--benzoylthymidine (IV) gave 3',5'-di-*O*-benzoyl-4-thiothymidine (V), amination of which with ethanolic anhydrous ammonia led to the isolation of 5-methyl-2'-de-oxycytidine (VIa) in 30% yield with respect to V. Amination of V under analogous conditions with ethanolic methylamine led to the desired N^4,5-dimethyl-2'-deoxy-cytidine (VIb), the properties of which were identical to those described by Fox *et al.* (1959). Finally treatment of V with ethylamine in ethanol under pressure at 120° gave the sought for N^4-ethyl-5-methyl-2'-deoxycytidine (VId), with properties similar to those of VIb.

The preparation of the dimethylamino analogue of VIa i.e. N^4,N^4,5-trimethyl--2'-deoxycytidine (VIc) presented special problems. Attempts to introduce a di-methylamino substituent in 2,4-dithiothymine (Russell, Elion, Falco & Hitchings, 1949) and 1-methyl-4-*O*-methylthymine (Shoup, Miles & Becker, 1967) were un-successful. Similar failures to introduce such a substituent into 3-methyl-4-thiouracil (Ueda & Fox, 1963) and 3-methyl-2,4-dithiouracil (Brown, 1959) suggested that the presence of a methyl substituent *ortho* to a 4-thio or 4-alkoxy offered appreciable steric hindrance to replacement of the latter by dimethylamine. This is easily seen by an examination of such structures with the aid of Koltun models which, however, demonstrate that under these conditions the dimethylamino group can be accommo-dated in the presence of a 3- or 5-methyl substituent if it is oriented in a plane per-pendicular to that of the ring. In view of the foregoing, attempts were continued with dimethylamine at various temperatures and pressures.

Treatment of V with anhydrous dimethylamine at 65° under nitrogen yielded only unreacted V. With 70% dimethylamine in anhydrous methanol at 120°, the products were thymidine (III) and the monomethylamino nucleoside (VIb). When, however, the amination reaction was carried out with 70% dimethylamine in an-hydrous methanol at 108 - 110° (2×40 hr.), the resultant reaction mixture contained thymidine together with an amino nucleoside and exhibited an absorption spectrum the principal absorption maximum of which was shifted bathochromically by 3 mμ with respect to VIb. This suggested the presence of some compound absorbing at longer wavelengths. The reaction mixture was consequently submitted to repeated chromatography on Whatman *3 MM* paper in solvent *E*, leading to the isolation of a minor component with a high R_F value (see Table 3). This compound was

eventually eluted and crystallized. In aqueous solvents it migrated above VIb, and its UV absorption maximum was 13 mμ to the red of VIb, while its pK_{a1} was slightly lower than that of VIb. Its absorption spectrum resembled that of N^4,N^4-dimethyl--5-fluoro-2′-deoxycytidine (Wempen, Duschinsky, Kaplan & Fox, 1961). At room temperature it was stable in 1 N-HCl (16 hr.) and in 33% aqueous dimethylamine (24 hr.). This compound appeared to be the desired $N^4,N^4,5$-trimethyl-2′-deoxycytidine (VIc).

Heating of VIc in anhydrous ethanol or methanol at 110 - 120° resulted in a gradual shift of the UV absorption maximum towards the violet, about 9 mμ in four days. Heating in aqueous dimethylamine at 120° led to gradual conversion of VIc to thymine; while heating at 120° in 1 N-HCl resulted in a gradual disappearance of the UV absorption spectrum and the appearance of unidentified products.

The fact that treatment of V with dimethylamine at elevated temperatures led to formation of VIb raises the suspicion that this may be due to the presence of methylamine in the commercial preparation of dimethylamine (VEB Berlin-Chemie, anhydrous, purest). Russell *et al.* (1949) had earlier reported that treatment of 2,4-dithiouracil with diamylamine gave unreacted product and N^4-monoamyl-2--thiopyrimidine, the presence of the latter being interpreted as due to contamination of the di-*n*-amylamine with *n*-amylamine. The possibility of dealkylation of VIc during the amination reaction by hydrolysis may be excluded in view of the anhydrous nature of the reaction medium, and the fact that hydrolysis in alkaline or acid media leads initially to deamination and, eventually, to fission of the glycosidic linkage.

Bearing in mind that, during dimethylamination, formation of monomethylamino derivatives may result in part from the presence of monomethylamine impurities, the appearance of VIb could conceivably be due to the formation of the intermediate VIe followed by the elimination of methylmercaptan, as illustrated in Scheme 2, whereas the elimination of H_2S from VIe would lead to VIc. From steric considerations one would expect the elimination of methylmercaptan to be favoured, with resultant higher yields of VIb as compared to VIc.

R = deoxyribose moiety
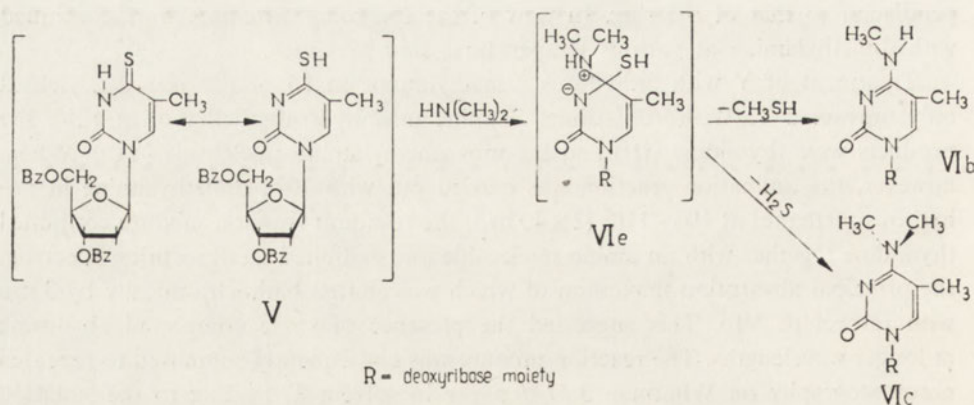
Scheme 2.

Although it is true that VIc was obtained by amination only in very low yield, it is nonetheless the first indication of the possibility of obtaining an N^4,N^4-dialkyl-aminonucleoside with an ortho methyl group by direct amination of the corresponding 4-thio analogue. The possibility of extending this procedure is considered again below.

Synthesis of aminopyrimidine analogues

These compounds, 1,5-dimethylcytosine and its 4-alkylamino derivatives (IIa, b, d), were prepared for purposes of comparison with the corresponding deoxy-ribosides. 1-Methylcytosine may be obtained by amination of 1-methyl-4-O-alkyl-uracil (Schmidt-Nickles & Johnson, 1930) or 1-methyl-4-thiouracil (Hilbert, 1934). Since, as mentioned above, attempts to dimethylaminate 4-alkoxy and 4-thio-5--methylpyrimidines were unsuccessful, it was decided to examine these in greater detail. Amination of 1-methyl-4-O-ethylthymine (I) proceeded less readily than that of the corresponding compound without a 5-methyl substituent, and required the use of higher temperatures to give the required 1,5-dimethylcytosine (IIa). The corresponding N^4-methyl and N^4-ethyl analogues (IIb, d) were obtained by treatment of the 4-alkoxy derivative I with the corresponding amines at 110 - 125°.

Treatment of I with dimethylamine, by contrast, led only to formation of 1--methylthymine (IIe), irrespective of temperature and time of the amination reaction under various conditions. This is undoubtedly due to the steric hindrance associated with the substitution of a dimethylamino group in a position ortho to the pyrimidine 5-methyl, as was placed in evidence by an examination of a Koltun model, and as previously referred to in attempts to aminate 4-thio (Russell *et al.*, 1949) and 4-ethoxy-5--substitued pyrimidines (Shoup *et al.*, 1967). The latter authors observed, during di-methylamination, dealkylation of 1-methyl-4-O-methylthymine to 1-methylthymine, both in aqueous medium and in anhydrous methanol. Hilbert & Johnson (1930) had earlier shown that, at elevated temperatures, 4-O-methyl derivatives of uracil and thymine undergo dealkylation and rearrangement much more readily than the corresponding 4-O-ethyl derivatives. It was consequently anticipated that the use of the 4-O-ethyl derivative (I) in the reaction with dimethylamine in anhydrous methanol would lead to formation of the required 1,N^4,N^4,5-tetramethylcytosine. However, all attempts to obtain this compound were unsuccessful. The failure to dimethylaminate compound I, and the widespread utility of the thiation method for the synthesis of aminopyrimidines and amino nucleosides, suggested the advisability of an examination of the amination of 1-methyl-4-thiothymine.

1-Methylthymine (IIe) was prepared by the hydrolysis of I, thiated with phospho-rous pentasulphide (Fox *et al.*, 1959) to give 1-methyl-4-thiothymine (IIf), and the latter treated with 60% dimethylamine in anhydrous ethanol at elevated tempera-tures to yield a compound with the spectral characteristics of 1,N^4,5-trimethyl-cytosine (IIb) (Fig. 1a). This was subjected to a second amination to yield a product
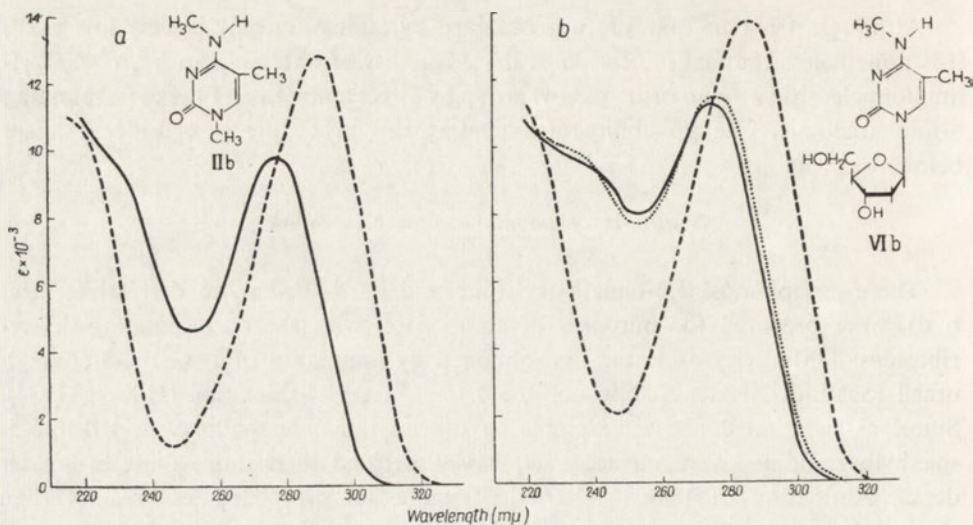
Fig. 1. Absorption spectra of (*a*), 1,N^4,5-trimethylcytosine, and (*b*), N^4,5-dimethyl-2′-deoxycytidine: (------), pH 1, protonated form; (———), pH 7 - 12, neutral form; (···), pH 14, showing effect of dissociation of sugar hydroxyl(s).
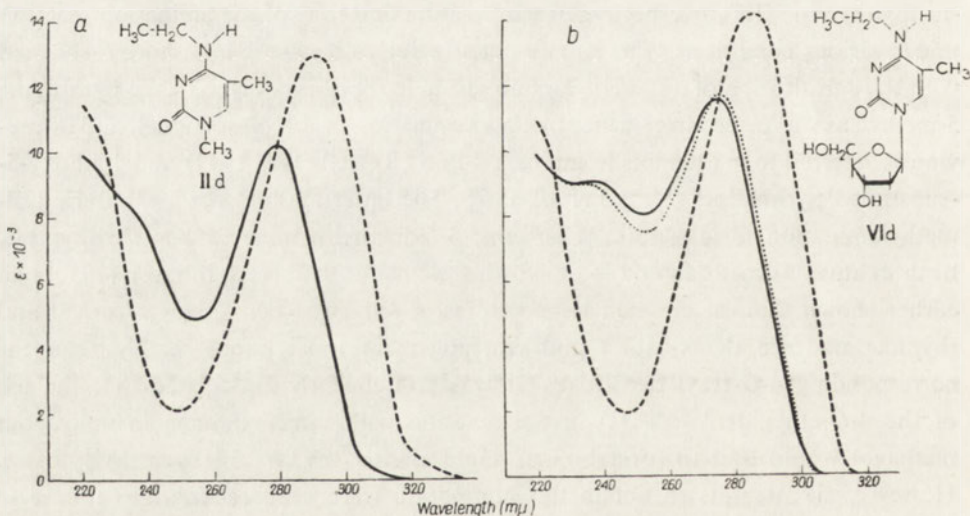

Fig. 2. Absorption spectra of (*a*), 1,5-dimethyl-N^4-ethylcytosine, and (*b*), N^4-ethyl-5-methyl-2′--deoxycytidine: (------), pH 1, protonated form; (———), pH 7 - 12, neutral form; (···), pH 14.

the UV absorption maximum of which was shifted to the red about 2 mμ relative to that of IIb. When this was chromatographed twice on Whatman no. 3 paper with solvent *A*, it yielded two components; the one with the smaller R_F exhibited the spectral characteristics of IIe; whereas that with the higher R_F value possessed

an absorption maximum at pH 1 at 290 mμ, with a pK_a of about 4.5. This latter is the monomethylamino derivative IIb.

It consequently appears that 4-O-alkylthymines are not suitable intermediates for the preparation of the corresponding $N^4,N^4,5$-trimethylpyrimidines. In the case of the nucleosides these analogues may be obtained, albeit in relatively low yield (see above) from the corresponding 4-thio derivatives. It is, of course, quite possible that the analogous pyrimidine derivatives may be attainable by dimethylamination of the 4-chloro analogues; the only 4-dimethylaminopyrimidines with a 5-methyl substituent hitherto reported were obtained by treatment with dimethylamine of 2-thio-4-chloro-5-methylpyrimidine and its 2-substituted derivatives (Brit. Patent 810, 846 Mar. 25, 1959), and 2,4-dichloro-5-methylpyrimidine (Koppel, Springer, Robins & Cheng, 1962).

UV absorption spectra and dissociation constants

The UV absorption spectra of the N^4-alkyl analogues of 5-methyl-2'-deoxy-cytidine and of 1,5-dimethylcytosine are illustrated in Figs. 1 through 3. Spectra were recorded at various pH values in the range 1 - 14 but, for simplicity, only the neutral and fully ionized forms are shown. The intermediate values were employed for estimations of pK_a at room temperature, and these are listed in Table 1 along with the data for other cytosine and deoxycytidine analogues, for purposes of comparison. For the dimethylamino derivative (Fig. 3) the quantity of material isolated (see Experimental) was insufficient to determine the extinction coefficient.

Table 1

Apparent pK_a values (to ±0.05 pH units) at 20° of 2'-deoxycytidine, 5-methyl-2'-deoxycytidine and a number of their analogues

Compound	pK_{a1}[a]	pK_{a2}[b]
1,5-Dimethylcytosine (IIa)	4.76[c]	—
1,N^4,5-Trimethylcytosine (IIb)	4.57	—
1,5-Dimethyl-N^4-ethylcytosine (IId)	4.58	—
5-Methyl-2'-deoxycytidine (VIa)	4.40[c]	13
N^4,5-Dimethyl-2'-deoxycytidine (VIb)	4.04[c]	13
N^4,N^4,5-Trimethyl-2'-deoxycytidine (VIc)	3.92	13
N^4-Ethyl-5-methyl-2'-deoxycytidine (VId)	4.05	13
1-Methylcytosine	4.55[d]	—
1,N^4-Dimethylcytosine	4.47[e]	—
1,N^4,N^4-Trimethylcytosine	4.20[e]	—
2'-Deoxycytidine	4.30[d]	13
N^4-Methyl-2'-deoxycytidine	4.01[f]	13
N^4,N^4-Dimethyl-2'-deoxycytidine	3.79[f]	13

[a] For protonation of ring N_3 nitrogen.
[b] For dissociation of carbohydrate hydroxyl(s).
[c] Data from Fox *et al.* (1959).
[d] Data from Fox & Shugar (1952).
[e] Data from Szer & Shugar (1966a).
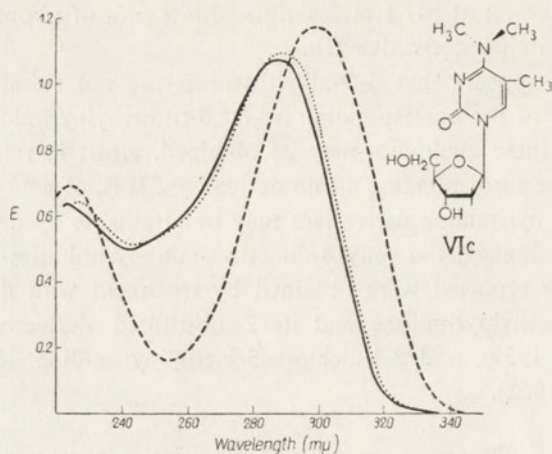[f] Data from Wempen *et al.* (1961).

Fig. 3. Absorption spectrum of N^4,N^4,5-trimethyl-2′-deoxycytidine: (------), pH 1, protonated form; (———), pH 7 - 12, neutral form; (···), pH 14.

Table 2 presents the shifts in wavelength and extinctions of the principal absorption maximum on introduction of one or two alkyl substituents in the exogenous amino group. Note that the introduction of the 5-methyl substituent gives the expected bathochromic shift but that this is accompanied by a decrease in the extinction coefficient. The introduction of a single methyl in the exogenous amino group provokes only a slight hypsochromic shift of the absorption maximum (about 0.5 mµ) and a marked increase in extinction. Introduction of an ethyl substituent further increases the extinction. Note, in particular, that the introduction of a second methyl in the exogenous amino group of the nucleoside markedly affects the shape of the neutral spectrum in the region 220 - 270 mµ. This is unquestionably related to the hindered rotation of the dimethylamino group which, as a result of the presence of the 5-methyl, probably lies in a plane perpendicular to that of the ring. It is consequently of interest that the neutral spectrum of N^4,N^4,5-trimethyl-2′--deoxycytidine (VIc, Fig. 3) so closely resembles that of N^4,N^4-dimethyl-5-fluoro--2′-deoxycytidine (Wempen *et al.*, 1961), where hindered rotation of the dimethylamino substituent might be expected to be less than with a 5-methyl substituent, and also bears some similarity to the spectrum of the neutral form of N^4,N^4-dimethyl-cytidine (Szer & Shugar, 1966a), for which the barrier to hindered rotation has been shown by means of NMR to be of the order of 8 kcal./mole (Martin & Reese, 1967).

It can also be seen from Fig. 3 that a 4-dimethylamino substituent appreciably reduces the extinction of the long wavelength absorption maximum of the protonated form of VIc with respect to that of the neutral form. In addition the extinction of the short wavelength maximum (about 227 mµ) is even more drastically reduced. This is most likely related to the additional hindrance to rotation of the dimethylamino group by the proton on N_3, an effect which has been experimentally demonstrated by Shoup *et al.* (1967) for the protonated form of 1,N^4-dimethylcytosine.

Table 2

Effect of alkylation of exogenous amino group on shift ($\Delta\lambda$ mμ) and change in extinction ($\Delta\varepsilon$) of absorption maxima of 2'-deoxycytidine, 5-methyl-2'-deoxycytidine, and some pyrimidine analogues

Compound	pH	λ_{max} (mμ)	$\Delta\lambda$ (mμ)	ε_{max} ($\times 10^{-3}$)	$\Delta\varepsilon_{max}$ ($\times 10^{-3}$)
1,5-Dimethylcytosine (IIa)	1	291	—	11.45	—
	7	280	—	7.77	—
1,N^4,5-Trimethylcytosine (IIb)	1	290.5	—0.5	12.70	+1.25
	7	278.5	—1.5	9.70	+1.93
1,5-Dimethyl-N^4-ethylcytosine (IId)	1	291	0.0	13.00	+1.55
	7	278.5	—1.5	10.10	+2.33
5-Methyl-2'-deoxycytidine (VIa)[d]	1	287	—	12.40	—
	7	277	—	8.50	—
N^4,5-Dimethyl-2'-deoxycytidine (VIb)	1	286.5	—0.5	13.60	+1.20
	7	275	—2.0	10.90	+2.40
N^4,N^4,5-Trimethyl-2'-deoxycytidine (VIc)	1	301	+14.0	—	—
	7	288	+11.0	—	—
N^4-Ethyl-5-methyl-2'-deoxycytidine (VId)	1	286.5	—0.5	14.04	+1.64
	7	275	—2.0	11.50	+3.00
1-Methylcytosine[a]	1	283	—	12.30	—
	7	274	—	8.15	—
1,N^4-Dimethylcytosine[b]	1	284	+1.0	12.38	+0.08
	7	274	—	8.90	+0.75
1,N^4,N^4-Trimethylcytosine[b]	1	288	+5.0	14.33	+2.03
	7	282	+8.0	11.30	+3.15
2'-Deoxycytidine[a]	1	280	—	13.20	—
	7	271	—	9.00	—
N^4-Methyl-2'-deoxycytidine[c]	1	282	+2.0	14.60	+1.40
	7	270	—1.0	11.70	+2.70
N^4,N^4-Dimethyl-2'-deoxycytidine[c]	1	287	+7.0	16.28	+3.08
	7	278	+7.0	14.05	+5.05

[a] Data from Fox & Shugar (1952).

[b] Data from Szer & Shugar (1966a).

[c] Data from Wempen *et al.* (1961).

[d] Data from Fox *et al.* (1959).

Figures 1b, 2b, and 3 also demonstrate for the alkylamino glycosides the effect of dissociation of the carbohydrate hydroxyl(s) on the absorption spectrum at pH above 12 (cf. Fox & Shugar, 1952).

Phosphorylation of nucleosides

The nucleosides described above were converted to the corresponding 5'-mono-phosphates by phosphorylation with 2-cyanoethylphosphate in the presence of dicyclohexylcarbodiimide (DCC) in anhydrous pyridine under the conditions previously described by Tener (1961) for the preparation of ^{32}P-labelled 5'-nucleotides from unprotected nucleosides. Phosphorylation of the unprotected nucleosides appeared to be the most convenient procedure after preliminary trials had indicated a yield of about 30%. This is certainly not less than the final yield from phosphorylation of the $N^4,O^{3'}$-diacetyldeoxycytidines since the acetylated nucleotides can be obtained with a yield of only 46% (Todd, 1957). Phosphorylation of the unprotected nucleosides is simpler and less arduous. Furthermore unreacted nucleoside is easily recovered, so that the overall yield is considerably better than with the protected compound.

The use of approximately 1 mole cyanoethylphosphate per mole nucleoside (Tener, 1961) led to formation of only minimal quantities of the nucleoside-3'-phosphates and prevented formation of the 3',5'-diphosphates. Only in the case of 5-methyl-2'-deoxycytidine was the ratio of phosphorylating agent to nucleoside increased, to 1:1, because of the formation of an amide with the pyrimidine exogenous amino group. Since the nucleosides VIa and VIb required a 20-fold larger volume of pyridine for complete solution, as compared to VId or 2'-deoxycytidine and thymidine, the quantity of dicyclohexylcarbodiimide was doubled. The isolation of the phosphorylation products was carried out essentially as described by Tener (1961).

Nucleoside-5'-triphosphates were then prepared as described by Moffatt (1964) with yields of about 60%. For the resulting triphosphates IXa, IXb and IXc, determinations of the ratio of phosphorus to extinction, P/ε, gave values of 3.25, 4.0 and 3.09, respectively, notwithstanding that final isolation involved chromatography on DEAE-cellulose columns; for the triphosphates of deoxycytidine and cytidine, this procedure was reported to give products with P/ε of 3 (Moffatt, 1964). The resulting impurities in our preparations were not removed even by adsorption and elution from activated charcoal (Żmudzka et al., in press). However, all three triphosphates proved to be substrates for the terminal transferase enzyme and yielded polymers with yields of more than 90% (Żmudzka et al., 1968).

EXPERIMENTAL

Melting points (uncorrected) were measured on a Beotius microscope hot stage. All absorption spectra were run on a Unicam SP 500 instrument, using 10-mm. cuvettes. For the measurement of the pK values of pyrimidine and nucleoside ana-

logues, Walpole acetate buffers were employed over pH range 2.5 to 5.5; Sörensen phosphate buffers in the range 6 to 8.6; 0.01 N and 0.1 N-HCl were taken as pH 2 and 1 respectively; 0.01 N, 0.1 N and 1N-NaOH as pH 12, 13 and 14. All pH measurements were made with a Radiometer PHM 22 instrument with glass semimicro electrodes.

Details regarding chromatography are given in Tables 1 and 3, a dark ultraviolet lamp being used to locate the spots on the paper. Inorganic phosphate was measured by the method of Fiske & Subba-Row (1925). Enzymic trials on nucleotides made use of *Crotalus adamanteus* snake venom as a source of 5'-nucleotidase (at pH 8.9 in the presence of Mg^{2+}), and prostate phosphomonoesterase (at pH 5.2).

1,5-Dimethylcytosine (IIa): This was obtained by amination of 200 mg. of 1--methyl-4-*O*-ethylthymine (I) (Schmidt-Nickles & Johnson, 1930) in ethanolic ammonia at 125° for 48 hr. Recrystallization of the product from anhydrous ethanol with addition of Norit gave long, colourless needles (111 mg., 65%) with m.p. 308 - 310°. Fox *et al.* (1959) report 308 - 310°.

1,N⁴,5-Trimethylcytosine (IIb): 200 mg. of I in 70 ml. of 45% methanolic methylamine was heated in a sealed tube for 40 hr. at 115 - 125°. Following removal of solvent, the residue was crystallized from ethyl acetate with addition of Norit to give 152 mg. (75%) with m.p. 177 - 180°. Following recrystallization from ethyl acetate the m.p. was 177 - 179°; Shoup *et al.* (1967) give 177 - 178°. Spectral data are shown in Fig. 1a.

1,5-Dimethyl-N⁴-ethylcytosine (IId): 200 mg. of I was dissolved in 3 ml. anhydrous ethanol to which was added 1.5 ml. anhydrous ethylamine and the solution heated in a sealed tube for 48 hr. at 125°. Removal of solvent, and crystallization of the residues from ethyl acetate gave 142 mg. (70%) with m.p. 139 - 141°. Recrystallization from ethyl acetate raised the m.p. to 144°. Spectral data are presented in Fig. 2a.

1-Methylthymine (IIe): 400 mg. of I in 10 ml. conc. HCl was heated under reflux for 6 hr. Solvent was removed, the residue was dissolved in a minimal quantity of water and left in the cold overnight. The crystals were removed on a sintered glass filter, washed with cold water and dried over P_2O_5 to give 300 mg. (81%) with m.p. 288 - 290°; Schmidt-Nickles & Johnson (1930) report 291°.

1-Methyl-4-thiothymine (IIf): 200 mg. of IIe was thiated with phosphorous pentasulphide in pyridine as described by Fox *et al.* (1959), to give 110 mg. (62%) with m.p. 211 - 213°.

Action of dimethylamine on 1-methyl-4-thiothymine (IIf): 80 mg. of IIf was dissolved in anhydrous 70% methanolic dimethylamine and heated in a sealed tube for 42 hr. at 105°. Solvent was removed and the residue chromatographed on Whatman 3 *MM* with solvents *A - E*, to give one spot with the properties of IIb. The residue was subjected to a second amination as above and, following removal of solvent, the residue was crystallized from methanol-acetone to give 22 mg. with m.p. 213 - - 217° (unreacted 4-thio compound). The filtrate was brought to dryness and the resulting residue chromatographed twice on Whatman 3 *MM* paper with solvent *A*

to give two spots. That with the lower R_F exhibited the spectral characteristics of IIe. The second spot possessed an absorption maximum at 290 mμ at pH 1 and turned out to be IIb.

Action of dimethylamine on 1-methyl-4-O-ethylthymine (I): 200 mg. of I was heated in a sealed tube for 48 hr. at 120° in 50% anhydrous methanolic dimethylamine. Solvent was removed and the residue crystallized from water with addition of Norit. The resulting crystals were recrystallized twice from water and ethanol to give a product with m.p. 287° and spectral characteristics as for IIe.

3′,5′-Di-O-benzoylthymidine (IV): This was prepared according to Wempen *et al.* (1963) to give a product with m.p. 193.5 - 195°, and 82% yield.

3′,5′-Di-O-benzoyl-4-thiothymidine (V): Prepared according to Wempen *et al.* (1963), yield 73%, m.p. 160.5 - 161°.

5-Methyl-2′-deoxycytidine (VIa): Obtained according to procedure of Wempen *et al.* (1963) in 42% yield, m.p. for monohydrate, 191 - 193° and for anhydrous hydrochloride salt 152 - 153.5°.

N^4,5-Dimethyl-2′-deoxycytidine (VIb): Prepared according to Fox *et al.* (1959) in 65% yield, m.p. 221 - 223°, and spectral data as in Fig. 1b.

N^4-Ethyl-5-methyl-2′-deoxycytidine (VId): 4.5 g. of V in 90 ml. of strictly anhydrous 60% ethanolic ethylamine was heated in an autoclave for 40 hr. at 105 - - 115°. Removal of solvent gave a syrup which was dissolved in 200 ml. water. The solution was reduced to one-half volume and extracted with chloroform (200, 100 and 100 ml.). The aqueous phase was treated with Norit and then brought to dryness to give a syrup which was dissolved in 30 ml. hot methanol, to which was added Norit, followed by heating to boiling for 5 min. and then filtered to give a colourless solution. The methanolic solution was brought to dryness, the residue dissolved in 25 ml. boiling ethanol, cooled to 40°, and ether added to a faint turbidity. The solution was left to cool slowly to room temperature with constant stirring and then left in the cold room overnight, to give a crystalline deposit, 1.21 g., m.p. 186 - 188°. The filtrate was warmed to 40° and a second crop of crystals brought down by addition of ether (0.5 g., total yield 60%). Following recrystallization from ethyl acetate, m.p. 217 - 219°. Spectral data, Fig. 2b.

Action of dimethylamine on 3′,5′-di-O-benzoyl-4-thiothymidine (V): A solution of 2.2 g. of V and 50 g. of anhydrous dimethylamine in 30 ml. anhydrous methanol was heated at 100 - 108° for 32 hr. Solvent was removed, the residue dissolved in 50 ml. water, and the latter brought to dryness. The residue was again dissolved in 50 ml. water and brought to dryness. The residue was once more dissolved in 50 ml. water, extracted 3× with 50 ml. portions of chloroform, the aqueous phase decolorized with Norit and brought to dryness, to give an oil. Half of this was dissolved in several ml. water, deposited on a 23×1.8 cm. Dowex 1X2 (OH⁻) column, and eluted with water (62 fractions of 20 ml.). Fractions 7 - 15 exhibited high absorption at 290 mμ; these were combined, brought to dryness, crystallized from anhydrous ethanol, and recrystallized overnight from ethanol to give 5 mg. of a product with m.p. 200° and λ_{max} of 287 mμ at pH 1. For $C_{11}H_{19}N_3O_5$ (mono-

hydrate of VIb), N (theor.) is 15.38%; N measured 15.30%. The filtrates were cooled to -5° to give a new crop of crystals, 160 mg., which, after recrystallization from methanol, had m.p. 217 - 220° and the spectral characteristics of VIb.

The remaining half of the syrup was subjected to a second treatment with dimethylamine as above. The residue remaining after removal of solvent was taken up in anhydrous hot ethanol. Stepwise addition of ether to the hot solution gave first a gummy precipitate, then an oil and finally some crystals. The crystals were subjected to chromatography on Whatman 3 *MM* with solvent *E*, in two runs of 16 hr. each, to give three spots. The one with the lowest R_F was found to be thymidine, the next was VIb, and that with the highest R_F (0.84, see Table 3) was eluted with anhydrous ethanol, the eluate brought to dryness, and the residue crystallized from ethanol-ether to give a small quantity of crystals which, after drying over P_2O_5, melted at 173 - 175°. For the monohydrate of VIc, $C_{12}H_{21}O_4N_3$, $N_{theor.}$ is 14.62% and $N_{exper.}$ 14.76%. Spectral data in Fig. 3.

5-Methyl-2'-deoxycytidine-5'-phosphate (VIIa): To 2 moles of VIa (482 mg.) dissolved in 10 ml. water was added 2 m-moles of 2-cyanoethylphosphate, followed by 20 ml. pyridine. The solution was concentrated to an oil and rendered anhydrous by three evaporations with dried pyridine. The residue was taken up in 220 ml. dried pyridine and 10 m-moles (2.06 g.) DCC added. The reaction mixture was shaken at room temperature for 24 hr., and the reaction interrupted by evaporation of the solution to about 60 ml. and addition of an equal volume of water. The resulting cyclohexylurea precipitate was filtered off, the filtrate concentrated to 10 ml., and 10 ml. conc. NH_4OH added. The mixture was then heated for 90 min. at 60°, the solvent evaporated off and the residue dissolved in 60 ml. water and heated for 45 min. at 100°. The solution was brought to room temperature and deposited on a column (2.1 × 12.5 cm.) of Dowex 1 X8, 200/400 mesh (formate form). The column was washed with water to remove unreacted nucleoside VIa (7 250 E units). Elution was then started with 0.02 M-HCOOH and 25-ml. fractions collected at 5-min. intervals. A small peak, between 250 and 300 ml., was discarded. 5-Methyl-2'-deoxycytidine-5'-phosphate (7 780 E units) was then eluted between 420 and 600 ml.; the eluate was concentrated under reduced pressure, and this procedure repeated with addition of ethanol. The concentrated alcohol-water solution was stored at 0° to induce crystallization. The crystals of VIIa were collected by centrifugation and washed with anhydrous ethanol and ether. The product, in the form of the monohydrate, was dried under vacuum over P_2O_5. Spectral estimation, on the basis of an ε of 12×10^3 at 287 mμ, pH 2, gave 212 mg. (0.63 m-mole, 31%). Phosphate determinations gave P/ε for the nucleotide 1.00:1.00. The product was chromatographically homogeneous in solvents *E, F, G* and *H* and was quantitatively converted to VIa on incubation with snake venom. UV absorption data: in 0.1 N-H_2SO_4 λ_{max} 287 mμ, λ_{min} 245 mμ; in 0.1 N-NaOH, λ_{max} 278 mμ, λ_{min} 254 mμ.

$N^4,5$-*Dimethyl-2'-deoxycytidine-5'-phosphate (VIIb):* 0.8 m-moles of VIb was phosphorylated as above with 0.4 m-moles cyanoethylphosphate and 4 m-moles

Table 3

R_F values of 5-methyl-2'-deoxycytidine and derivatives

Ascending paper chromatography on Whatman no. 1 of 5-methyl-2'-deoxycytidine and some of its structural analogues, with the following solvent systems (ratios in all cases v/v): A: water--saturated butanol; B: isopropanol - water - conc. HCl (d = 1.18), 65:18.4:16.6; C: tert. butanol-- methylethylketone - NH_4OH (d = 0.88) - water, 4:3:1:2; D: ethyl acetate - formic acid - water, 70:20:10; E: isopropanol - conc. NH_4OH - water, 7:1:2; F: 95% ethanol - 1 M-ammonium acetate, 5:2; G: isobutyric acid - 1 M-NH_4OH - 0.1 M-EDTA (pH = 8.2), 100:60:1.6; H: n-propanol - conc. NH_4OH - water, 6:3:1.

Compound	R_F in solvent							
	A	B	C	D	E	F	G	H
Cytosine	0.13	0.46	0.58	0.52	0.61	0.67		
1,5-Dimethylcytosine (IIa)	0.30	0.59	0.70	0.69	0.67	0.84		
1,N^4,5-Trimethylcytosine (IIb)	0.54	0.64	0.81	0.76	0.79	0.92		
1,5-Dimethyl-N^4-ethylcytosine (IId)	0.66	0.83	0.89	0.86	0.92	0.94		
5-Methyl-2'-deoxycytidine (VIa)	0.30	0.55	0.70	0.52	0.66	0.82		
N^4,5-Dimethyl-2'-deoxycytidine (VIb)	0.45	0.62	0.80	0.61	0.79	0.92		
N^4,N^4,5-Trimethyl-2'-deoxycytidine (VIc)	—	0.64	—	0.75	0.84	—		
N^4-Ethyl-5-methyl-2'-deoxycytidine (VId)	0.57	0.80	0.89	0.86	0.90	0.94		
5-Methyl-2'-deoxycytidine-5'-phosphate (VIIa)					0.11	0.25	0.66	0.42
N^4,5-Dimethyl-2'-deoxycytidine-5'-phosphate (VIIb)					0.18	0.41	0.58	0.47
N^4-Ethyl-5-methyl-2'-deoxycytidine-5'--phosphate (VIIc)					0.23	0.54	0.68	0.53
5-Methyl-2'-deoxycytidine-5'-phosphoro-morpholidate (VIIIa)					0.56		0.81	0.68
N^4,5-Dimethyl-2'-deoxycytidine-5'--phosphoromorpholidate (VIIIb)					0.66		0.74	0.79
N^4-Ethyl-5-methyl-2'-deoxycytidine-5'--phosphoromorpholidate (VIIIc)					0.74		0.84	0.84
5-Methyl-2'-deoxycytidine-5'-diphosphate							0.54	0.37
N^4,5-Dimethyl-2'-deoxycytidine-5'--diphosphate							0.48	0.38
N^4-Ethyl-5-methyl-2'-deoxycytidine-5'--diphosphate							0.58	0.45
5-Methyl-2'-deoxycytidine-5'-triphosphate (IXa)							0.44	0.32
N^4,5-Dimethyl-2'-deoxycytidine-5'--triphosphate (IXb)							0.35	0.31
N^4-Ethyl-5-methyl-2'-deoxycytidine-5'--triphosphate (IXc)							0.44	0.38

(824 mg.) DCC in 120 ml. pyridine. Isolation of the product was carried out as for VIIa, above. Washing the column with water removed 6 350 E units of unreacted nucleoside (VIb). The nucleotide VIIb (2 450 E units) was eluted in the fractions between 350 and 600 ml. 0.02 M-HCOOH. Two small peaks (100 E units) which appeared before VIIb were discarded. The fractions containing VIIb were con-

centrated, evaporated from ethanol, and VIIb precipitated from an aqueous ethanolic solution by addition of ether, and washed with ethanol to give 64.3 mg. (0.18 m-mole by spectral estimation, yield 22%), ratio of P to ε 1.06:1. The product was chromatographically homogeneous in solvents E, F, G, H and was quantitatively converted to VIb by snake venom. UV absorption: in 0.1 N-H_2SO_4, λ_{max} 286 mμ, λ_{min} 245 mμ; in 0.1 N-NaOH, λ_{max} 275 mμ, λ_{min} 252 mμ.

N^4-*Ethyl-5-methyl-2'-deoxycytidine-5'-phosphate (VIIc):* 2 m-moles of VId (538 mg.) was phosphorylated with 1 m-mole cyanoethylphosphate and 4 m-moles (824 mg.) DCC in 12 ml. pyridine, as above. Phosphorylation was interrupted after 2 days by addition of 12 ml. water. Conc. NH_4OH (24 ml.) was added, the solution heated 90 min. at 60° and reduced to dryness under vacuum. The filtrate was deposited on a column (2.1 × 16 cm.) of Dowex 1 X8, 200/400 mesh (formate form). Washing with water removed unreacted VId (13 000 E units). Elution with 0.02 M-HCOOH removed the nucleotide (5 040 E units) in the fractions between 300 and 500 ml. Continued elution with the same solvent gave the 3'-phosphate of VId (480 E units) between 540 and 700 ml. The fractions included between 140 - - 280 ml. removed from the column a compound (1 590 E units) with an R_F of 0.62 in the solvent system E which contained phosphate (identified by spraying) and was resistant to snake venom and non-specific phosphomonoesterase; this compound was not identified. The fractions containing the nucleotide VIIc were concentrated under reduced pressure and precipitated from an aqueous ethanolic solution with ether to give an oil which, on trituration with ether, was transformed to a hygroscopic precipitate (141 mg., 0.37 m-mole by spectral estimation, 19% yield). The ratio of P to extinction was 0.94:1 and the product was homogeneous with solvents A, B, C, D and quantitatively converted to VId with snake venom. UV absorption: in 0.1 N-H_2SO_4, λ_{max} 287 mμ, λ_{min} 247 mμ; in 0.1 N-NaOH λ_{max} 276 mμ, λ_{min} 252 mμ.

Nucleoside-5'-phosphoromorpholidates (VIIIa,b,c): All three of the above nucleoside-5'-phosphates were converted to the nucleoside-5'-phosphoromorpholidates (isolated as the 4-morpholine-N,N'-dicyclohexylcarboxamidine salts) by slight modifications of the method of Moffatt & Khorana (1961). Starting quantities and yields are presented in Table 4.

5-Methyl-2'-deoxycytidine-5'-triphosphate (IXa): 171 mg. (0.25 m-mole) of the 4-morpholine-N,N'-dicyclohexylcarboxamidinium salt of VIIIa was dried by two

Table 4

Yields of nucleoside 5'-phosphoromorpholidates from nucleoside 5'-phosphates

Nucleotide	Amount	Yield of 5'-phosphoromorpholidate
VIIa	160 mg. (0.5 m-mole)	VIIIa: 347 mg., 0.48 m-mole, 94%
VIIb	59 mg. (0.17 m-mole)	VIIIb: 107 mg., 0.13 m-mole, 77%
VIIc	108 mg. (0.3 m-mole)	VIIIc: 230 mg., 0.27 m-mole, 80%

evaporations from 10 ml. portions of anhydrous pyridine, followed by two evaporations from 5-ml. portions of anhydrous benzene. In a separate flask, 1.0 m-mole tetrasodium pyrophosphate ($10 \cdot H_2O$) was converted to the tri-n-butylamine salt (Moffatt, 1964) and rendered anhydrous by four evaporations with 10-ml. portions of dried benzene. The product was added to the dried morpholidate with the aid of four 1-ml. portions of dimethyl sulphoxide (which had been dried by distillation and storage for several days over Type 4A molecular sieve, a product of the Inowrocław Soda Plant, Poland). The solution was kept at room temperature for two days, 30 ml. water added, and the whole applied to a 2×35 cm. column of DEAE-cellulose (Whatman DE-50) in the bicarbonate form. The column was washed with water until the effluent was free of UV absorption. The products were then eluted with a linear gradient of triethylammonium bicarbonate (1500 ml. water, 1500 ml. 0.35 M-triethylammonium bicarbonate). Four clearly resolved UV-absorbing peaks were collected. The last, which was the triphosphate IXa, was brought to dryness at 30 - 35° with a rotary evaporator, the collecting bulb being kept at -10° to -20°. Residual triethylammonium bicarbonate was removed by four evaporations with 25-ml. portions of methanol. The residue was dissolved in 5 ml. methanol (0.176 m--mole, estimated spectrally) to which was added 0.078 ml. of 1 M-sodium iodide (1.06 m-moles) in acetone and then 75 ml. acetone. The resulting precipitate was collected by centrifugation, washed three times with 30-ml. portions of acetone and dried overnight under vacuum (123 mg., 71% yield); ratio of P to extinction 3.25:1, chromatographically homogeneous in solvents G and H, UV absorption characteristics similar to VIIa.

$N^4,5$-*Dimethyl-2'-deoxycytidine-5'-triphosphate (IXb):* This was prepared from 87.25 mg. (0.108 m-mole) of the 4-morpholine-N,N'-dicyclohexylcarboxamidinium salt of VIIIb and 0.43 m-mole of the tri-n-butylamine salt of pyrophosphate under conditions analogous to those for IXa. The triphosphate fraction from the column yielded 0.05 m-mole (46%) estimated spectrally. It was not fully homogeneous on paper chromatography with solvents G and H, and the molar ratio of phosphate to extinction was 4.00:1. However, the absorption spectrum in acid and alkali was similar to that for VIIb.

N^4-*Ethyl-5-methyl-2'-deoxycytidine-5'-triphosphate (IXc):* Prepared from 142 mg. (0.2 m-mole) of the 4-morpholine-N,N'-dicyclohexylcarboxamidinium salt of VIIIc and 0.8 m-mole of the tri-n-butylamine salt of pyrophosphate, as for the preparation of IXa, to yield 79 mg. of the tetrasodium salt of VId (0.13 m-mole, 65%, in agreement with spectral estimation). Paper chromatography with solvent G demonstrated the presence of traces of the pyrophosphate, while the absorption spectrum coincided with that for VIIc in acid and alkali media.

REFERENCES

Brown D. J. (1959). *J. Appl. Chem.* **9**, 203.
Fiske C. H. & Subba-Row Y. (1925). *J. Biol. Chem.* **66**, 275.
Fox J. J. & Shugar D. (1952). *Biochim. Biophys. Acta* **9**, 369.

Fox J. J., Van Praag D., Wempen I., Doerr I. L., Cheong L., Knoll J. E., Eidinoff M. L., Bendich A. & Brown G. B. (1959). *J. Amer. Chem. Soc.* **81**, 178.

Hilbert G. E. (1934). *J. Amer. Chem. Soc.* **56**, 190.

Hilbert G. E. & Johnson T. B. (1930). *J. Amer. Chem. Soc.* **52**, 2001.

Koppel H. C., Springer R. H., Robins R. K. & Cheng C. C (1962). *J. Org. Chem.* **27**, 181.

Kuo T. T., Huang T. C. & Tang M. H. (1968). *J. Mol. Biol.* **34**, 373.

Martin D. M. G. & Reese C. B. (1967). *Chem. Commun.* 1275.

Moffatt J. G. (1964). *Canad. J. Chem.* **42**, 599.

Moffatt J. G. & Khorana H. G. (1961). *J. Amer. Chem. Soc.* **83**, 649.

Prystaš M., Farkaš J. & Šorm F. (1965). *Coll. Czechoslov. Chem. Commun.* **30**, 3123.

Russell P. B., Elion G. B., Falco E. A. & Hitchings G. H. (1949). *J. Amer. Chem. Soc.* **71**, 2279.

Schmidt-Nickles W. & Johnson T. B. (1930). *J. Amer. Chem. Soc.* **52**, 4511.

Shoup R. R., Miles H. T. & Becker E. D. (1967). *J. Amer. Chem. Soc.* **89**, 6200.

Szer W. & Shugar D. (1966a). *Acta Biochim. Polon.* **13**, 177.

Szer W. & Shugar D. (1966b). *J. Mol. Biol.* **17**, 174.

Tener G. M. (1961). *J. Amer. Chem. Soc.* **83**, 159.

Todd A. R. (1957). In *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 811. Academic Press, New York

Ueda T. & Fox J. J. (1963). *J. Amer. Chem. Soc.* **85**, 4024.

Wempen I., Duschinsky R., Kaplan L. & Fox J. J. (1961). *J. Amer. Chem. Soc.* **83**, 4755.

Wempen I., Ueda T. & Fox J. J. (1963). *Biochem. Prep.* **10**, 98.

Żmudzka B., Bollum F. J. & Shugar D. (1968). *FEBS Abstracts, Praha.*

OTRZYMYWANIE I WŁASNOŚCI N^4-ALKILO ANALOGÓW 5-METYLO-2'-DEZOKSYCYTYDYNY, ICH 5'-MONO I TRÓJFOSFORANÓW ORAZ N^4-ALKILOPOCHODNYCH 1,5-DWUMETYLOCYTOZYNY

Streszczenie

1. Opisano syntezę i własności N^4-monometylo- i etyloamino analogów 5-metylo-2'-dezoksycytydyny i 1,5-dwumetylocytozyny oraz N^4,N^4-dwumetyloamino analogu 5-metylo-2'-dezoksycytydyny.

2. Szczególną uwagę poświęcono problemom związanym z otrzymywaniem N^4,N^4-dwumetyloamino analogów: zbadano działanie dwumetyloaminy na 1-metylo-4-O-etylotyminę, 1-metylo--4-tiotyminę oraz 3',5'-dwu-O-benzoilo-4-tiotymidynę.

3. Podano i przedyskutowano widma absorpcyjne w nadfiolecie i wartości pK_a szeregu 1-podstawionych N^4-alkilocytozyn.

4. Opisano chemiczną syntezę 5'-mono- i trójfosforanów 5-metylo-2'-dezoksycytydyny i ich alkiloamino analogów. W innej publikacji wykazano, że trójfosforany te są substratami dla transferazy dezoksynukleotydylowej.

CELINA JANION and D. SHUGAR

MECHANISM OF HYDROXYLAMINE MUTAGENESIS: COMPLEXING PROPERTIES OF COPOLYMERS OF HYDROXYCYTIDYLIC ACID WITH CYTIDYLIC OR URIDYLIC ACIDS

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Rakowiecka 36, Warszawa 12; and Department of Biophysics, University of Warsaw, Warszawa

1. Two series of copolymers have been prepared with the aid of polynucleotide phosphorylase, one of these containing a mixture of uridine and N^4-hydroxycytidine residues, the other a mixture of cytidine and N^4-hydroxycytidine residues. The properties of these copolymers are described. 2. Complexes of the foregoing polymers with poly-A and poly-I, respectively, were formed and their properties examined. 3. In particular, it was found that N^4-hydroxycytidine residues in the respective copolymers would not base-pair with either adenosine residues in poly-A or inosine residues in poly-I. 4. Copolymers of cytidine and N^4-hydroxycytidine were still capable of forming a twin-stranded "acid" helix similar to that of acid poly-C, but without participation of the N^4-hydroxycytidine residues. 5. The overall results are in accord with those previously obtained with the use of homopolymers, viz. N^4-hydroxycytidine residues do not base pair with either A or I (and U or C). 6. It is concluded that hydroxylamine mutagenesis involves more than simple base-pair transitions and that other factors, e.g. the specificity of the replicating enzymes, are probably also involved.

Of the known chemical mutagens, hydroxylamine ($\overline{HA}$[1], as well as its *O*-methyl and *N*-methyl analogues) has commanded special attention because of its specificity and resultant ability to induce point mutations. Both analyses of hydrolysates of hydroxylamine-treated nucleic acids, as well as the mutations resulting from treatment with high hydroxylamine concentrations, indicate that the mutagenic activity of hydroxylamine is due to some modification of cytosine residues (Freese *et al.*, 1961a, b; Freese & Strack, 1962; Schuster, 1963; Tessman, Poddar & Kumar, 1964; Brown

---

[1] The following abbreviations are used throughout this text: $\overline{HA}$, hydroxylamine, $\overline{HAC}$, N^4--hydroxycytidine; Hx, hypoxanthine; Cp, cytidylic acid; 3MeU, 3-methyluridine; poly-$\overline{HAC}$, poly-N^4-hydroxycytidylic acid; poly-U, poly-uridylic acid; poly-A, poly-adenylic acid; poly-C, poly-cytidylic acid; poly-I, poly-inosinic acid; poly-(A:U), twin-stranded helical complex of poly-A and poly-U; poly-(I:C), twin-stranded helix of poly-I and poly-C; poly-(U,C), single-stranded copolymer containing U and C residues, with the same connotations for other copolymers containing two different residues.

& Schell, 1965). The high mutagenic activity of hydroxylamine against the T-even bacteriophages has also been related to modification of the 5-hydroxymethylcytosine residues of their DNA (Freese *et al.*, 1961a,b; Champe & Benzer, 1962).

Despite the large number of investigations designed to elucidate the mechanism of hydroxylamine mutagenesis, there is still some controversy as to which of the modifications of the cytosine residues is mutagenic (Freese, 1963; Phillips, Brown, Adman & Grossman, 1965; Janion & Shugar, 1965b). Likewise open to question is the manner in which the modified cytosine residues lead to the expression of mutations (Freese, 1963; Phillips, Brown & Grossman, 1966; Nagata & Mårtensson, 1968).

The products resulting from the action of NH_2OH on cytosine, or a 5-substituted cytosine (or their glycosides) are illustrated in Scheme 1:
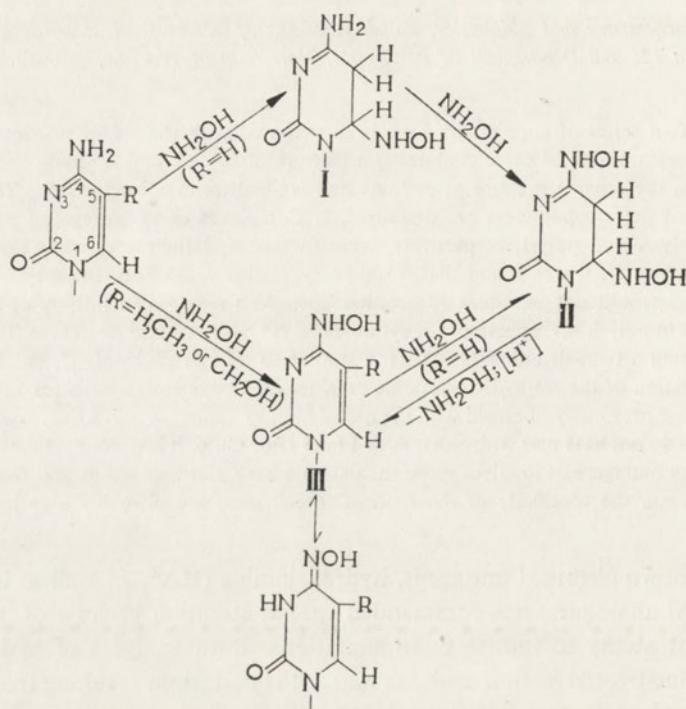
Scheme 1.

Although compound I is a presumed intermediate in the formation of II (Brown & Schell, 1961), and has not been isolated because of its suspected instability, indirect evidence has been adduced for its involvement in the mutagenic process (Brown & Phillips, 1965; Phillips *et al.*, 1965, 1966). Product II, which may be isolated directly from the reaction mixture, has also been implicated (Freese, 1963). Product III was for some time regarded as a by-product of II, resulting from the elimination of hydroxylamine from the 5,6 bond of II under acid conditions, although its potential role in mutagenesis was not entirely excluded.

The finding that III is the only reaction product with 5-substituted cytosines (Janion & Shugar, 1965a); and the subsequent demonstration that III is formed, albeit in lower yield, simultaneously with II, from the reaction between hydroxylamine and cytosine (Lawley, 1967; Kochetkov *et al.*, 1967; Janion & Shugar, 1968; Brown & Hewlins, 1968) has furnished a potent argument in favour of III as the source of mutations not only in the case of the T-even bacteriophages, but also with other biologically active systems such as transforming DNA (Freese & Strack, 1962; Bautz-Freese & Freese, 1964) and bacteriophage S13 (Tessman *et al.*, 1964), which contain cytosine in their DNA.

According to the classification of point mutations by Freese (1959), mutations due to the action of hydroxylamine were regarded as due to substitutions involving the transition of a G-C base pair to A-T, the transition involving a modification of the pairing properties of the modified cytosine. The latter was presumed to lose its ability to pair with guanine, and to do so with adenine (Freese *et al.*, 1961a). This modification in base-pairing ability has been variously ascribed to each of the three compounds formed from hydroxylamine-treated cytosine shown in Scheme 1, or to their tautomeric (i.e. 4-imino, or 4-oximino) forms.

General support for the above derives from experiments in which template properties of poly-C were examined following treatment of the homopolymer with NH_2OH. In the *in vitro* RNA polymerase system, poly-C directs the incorporation of GTP to form the complementary poly-G. Treatment of the poly-C with hydroxylamine leads to a loss in ability to direct the incorporation of GTP. On addition of ATP, but not UTP or CTP, the template properties of the NH_2OH-treated poly-C are partially restored, with the concomitant incorporation of ATP (Phillips *et al.*, 1965; Wilson & Caicuts, 1966).

In a previous study (Janion & Shugar, 1968) attempts were made to establish some physico-chemical confirmation for the foregoing findings by examining the modifications in base-pairing of the altered cytosine residues in NH_2OH-treated poly-C. Homopolymers were prepared containing either II or III as base residues and an examination was made of the ability of these polymers to complex with other potentially complementary homopolymers. Surprisingly, neither of the homopolymers proved capable of complexing with poly-A, nor did they appear to interact with poly-I, poly-C or poly-U.

These findings have now been extended to an examination of the complexing properties of copolymers containing random sequences of the residue III in various proportions with U or C. The results obtained are in agreement with those previously reported and suggest the need for a revision of present concepts regarding the mechanism of hydroxylamine mutagenesis.

MATERIALS AND METHODS

The sodium salt of CDP was obtained from Koch-Light (Colnbrook, England) and the lithium salt of UDP from Schwarz Bio-Research (Orangeburg, N.Y., U.S.A.); HA-CDP (the 5'-pyrophosphate of III) was prepared from CDP as previously

described (Janion & Shugar, 1968). Poly-U, poly-C, poly-I and poly-A were commercial preparations obtained from Miles Chemical Co. and Sigma Chemical Co. (St. Louis, Mo., U.S.A).

Spectral measurements and transition profiles were run on a Unicam SP 500 instrument fitted with a specially constructed copper-jacketed cuvette carriage, the temperature of which was controlled by means of a water-glycol mixture from a Hoeppler ultrathermostat over the range $2 - 90°$. Temperatures were measured by means of a calibrated thermistor. All pH measurements were made with a glass electrode and a Radiometer PHM-28 meter. Glass-distilled water was used throughout.

Mixing curves were obtained by preparing solutions of the polymers at equal molar concentrations and mixing these in various proportions. Complex formation between poly-I and copolymers of C and $\overline{HAC}$, poly-(C,$\overline{HAC}$), was appreciably slower than between homopolymers so that, following mixing, the resultant hypochromicity accompanying complex formation was estimated after a suitable time interval which varied from 15 min. to 2 hr., depending on the $\overline{HAC}$ content of the copolymer component. The rate of complex formation between poly-A and poly--(U,$\overline{HAC}$) did not differ significantly from that for formation of a complex between poly-A and poly-U. The measured change in absorption of a given mixture (in %) is the difference between the arithmetical sum of the components and that actually observed. The arithmetic sum of the components was calculated from

$$\frac{E_s \cdot V}{V_f} + \frac{E_s' \cdot V'}{V_f}$$

where E_s (E_s') is the absorption of a given component, V (or V') the volume of the component solution, and V_f the final volume of the mixture.

RESULTS

Synthesis of copolymers. Two preparations of polynucleotide phosphorylase (EC 2.7.7.8) were employed for copolymer syntheses. The *Micrococcus lysodeikticus* enzyme, kindly supplied by Dr. H. Matthaei, and consisting of fraction III from a purification procedure elsewhere described (Matthaei *et al.*, 1967), was used for preparation of poly-(C,$\overline{HAC}$). The *E. coli* enzyme (Kimhi & Littauer, 1968), a gift from Dr. U. Z. Littauer, was used to prepare poly-(U,$\overline{HAC}$).

The incubation mixture for the preparation of poly-(C,$\overline{HAC}$) included: 90 µl. of 0.5 M-tris buffer, pH 8.8, 30 µl. of 0.1 M-MgCl₂, 30 µl. of 3 M-NaCl, 12 µl. of 0.01 M-EDTA, 6 mg. of desired proportions of CDP and $\overline{HAC}$DP, 24 µl. enzyme, and water to a final volume of 300 µl. Incubation time at 37° was about 2 hr., and polymer yields varied from 17 - 32%.

The preparation of poly-(U,$\overline{HAC}$), purification of polymers, and calculation of yields were as described elsewhere for poly-$\overline{HAC}$ (Janion & Shugar, 1968). Yields of poly-(U,$\overline{HAC}$) varied from 25 - 35%. Polymer purification included several

deproteinizations with phenol, prolonged dialysis against decreasing salt concentrations in the presence of 0.01 M-EDTA, and finally against glass-distilled water. For both types of copolymers the ratio of the base residues did not differ visibly from the ratio of the pyrophosphate substrates in the incubation medium.

Polymer hydrolysis. Poly-I and poly-A were hydrolysed to mononucleotides in 1 N-NaOH at room temperature for 24 hr. The pyrimidine polymers and copolymers were exhaustively hydrolysed with pancreatic ribonuclease (EC 2.7.7.16). The compositions of the copolymers were obtained by chromatography of the enzymic hydrolysates and eluting with 0.1 N-HCl, using the following extinction coefficients: Up, 10.1×10^3; Cp, 8.9×10^3; $\overline{HAC}$p, 12.2×10^3. With ascending chromatography on Whatman paper no. 1, and the solvent system butanol - methanol - - 5 M-HCl (3:2:2, by vol.), the R_F values of Up, Cp and $\overline{HAC}$p were, respectively, 0.69, 0.44 and 0.51. Two-dimensional chromatography was occasionally employed to obtain better separations of Cp and $\overline{HAC}$p.

Copolymers of C and $\overline{HAC}$. The hyperchromicity resulting from hydrolysis to monomers of several copolymers is exhibited in Table 1. It will be seen that an increase in the number of $\overline{HAC}$ residues in the copolymer leads to a decrease in hyperchromicity. This is in agreement with the low hyperchromicity of poly-$\overline{HAC}$ itself at 275 mμ (Janion & Shugar, 1968). The neutral form of poly-C is known to be single-stranded, the hyperchromicity being a reflection of the degree of stacking of the bases (Fasman, Lindblow & Grossman, 1964). The pronounced decrease in hyperchromicity resulting from replacement of some of the cytosine residues by $\overline{HAC}$ would appear to imply the absence of interaction between C and $\overline{HAC}$ residues, but such a conclusion is perhaps equivocal. It will probably be necessary to examine such polymers by other methods, such as circular dichroism or optical

Table 1

Hyperchromicity accompanying enzymic hydrolysis to mononucleotides of copolymers of Cp and $\overline{HAC}$p, in 0.01 M-tris buffer, pH 7.7

Preparation no.	Content of HACp (%)	Hyper-chromicity (%) at 270 mμ
—	0	35
9	26	24.6
10	20	23.8
11	40	15.3
—	100	2.0

rotatory dispersion before final conclusions can be drawn. Figs. 1a and b exhibit typical absorption spectra of two preparations of poly-(C,$\overline{HAC}$) prior to and following hydrolysis by ribonuclease. Note, in particular, that the maximum at about 230 mμ,

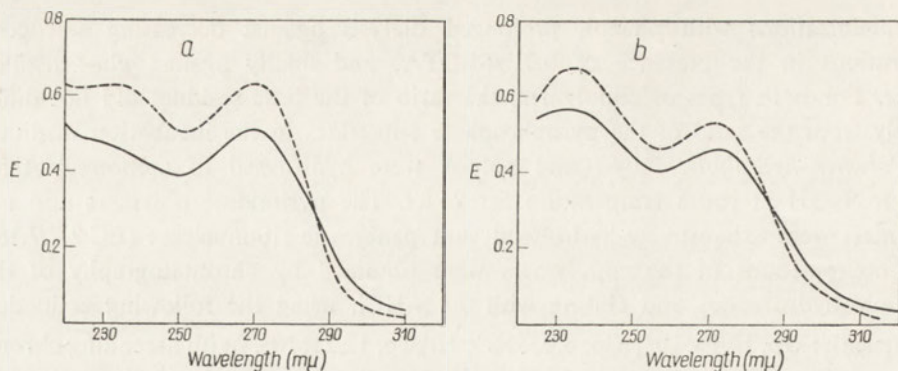Fig. 1. UV absorption spectra, in 0.01 M-tris buffer, pH 7.7, of copolymers of C and $\overline{HAC}$. (———) prior to, and (------) following hydrolysis to monomers with 20 μg./ml. pancreatic ribonuclease at 37° for 2 hr.: (a), poly-(C,$\overline{HAC}$) with $\overline{HAC}$ content of 20%; (b), 40% content of $\overline{HAC}$.

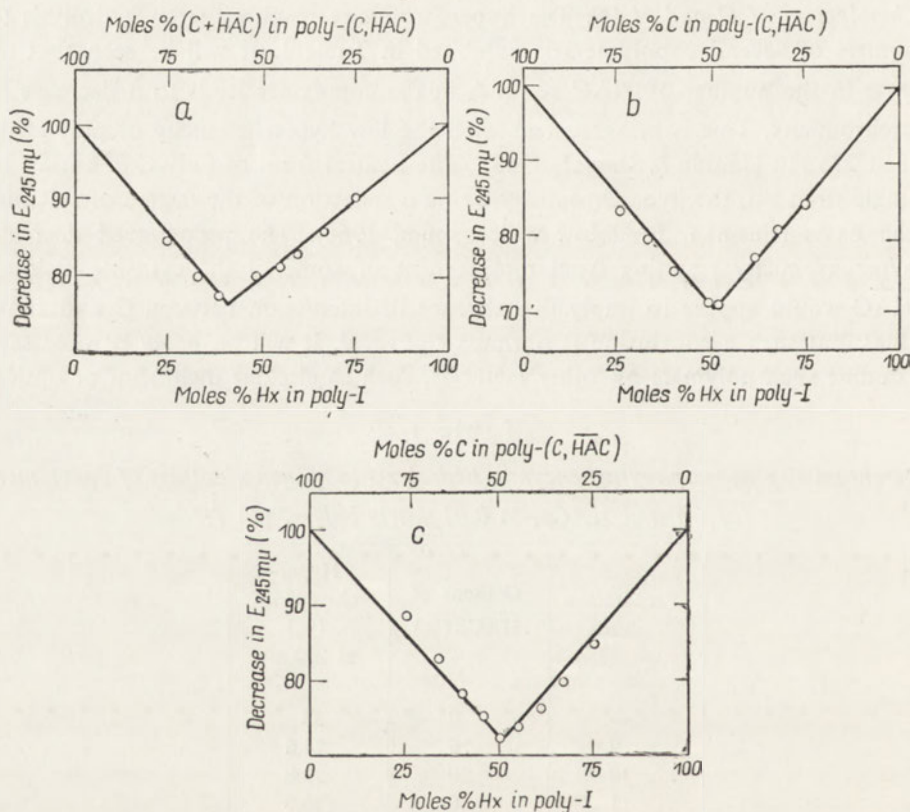Fig. 2. Mixing curves for poly-(C,$\overline{HAC}$) with poly-I ĩn 0.1 M-NaCl and 0.01 M-phosphate buffer, pH 7: (a), for copolymer containing 20% $\overline{HAC}$ residues, and for which the number of $\overline{HAC}$+C residues was taken equal to the number of Hx residues; (b), as in (a), but the number of C residues in the copolymer equated to the number of Hx residues in poly-I; (c), for copolymer containing 26% $\overline{HAC}$ residues and the number of C residues equated to the number of Hx residues in poly-I.

characteristic of the $\overline{HAC}$ monomer (compound III), becomes well defined on hydrolysis (cf. Janion & Shugar, 1965b).

Complexes of poly-(C,$\overline{HAC}$) with poly-I. These were studied with the aid of two copolymer solutions of different concentrations. In one of these the total concentration of pyrimidine residues was equal to the number of hypoxanthine residues in poly-I, i.e. (C+$\overline{HAC}$) : Hx was 1:1. In the second only the number of cytosine residues was taken into account so that C : Hx was 1:1. The mixing curves shown in Figs. 2a, b and c clearly demonstrate that maximum complex formation is attained with a ratio of C : Hx of 1:1. It follows that the $\overline{HAC}$ residues in the copolymers do not participate in complex formation and are probably forced out of the resulting helix in the form of loops, such as those formed in complexes of poly-A with the copolymer poly-(U,A) (Fresco & Alberts, 1960), of poly-A with the copolymer poly-(U,3MeU) (Szer & Shugar, 1961), and poly-I with poly-(C,4-dimethylC) (Brimacombe & Reese, 1966).

Attention should be directed to one source of error in the calculation of the hypochromicity accompanying complex formation when one of the components is a copolymer containing non-complementary base residues, viz., the latter do not contribute directly to the hypochromicity of the complex formed. Finally the absorption of the non-complementary base residues themselves should not be taken into account in calculations of the overall hypochromicity; but this is difficult to correct for, and was not done since it does not affect the conclusions arising from the mixing experiments.

Thermal dissociation of poly-(I:C,$\overline{HAC}$). Melting profiles for poly-(I:C,$\overline{HAC}$) are shown in Fig. 3 for several complexes with different contents of $\overline{HAC}$ residues.
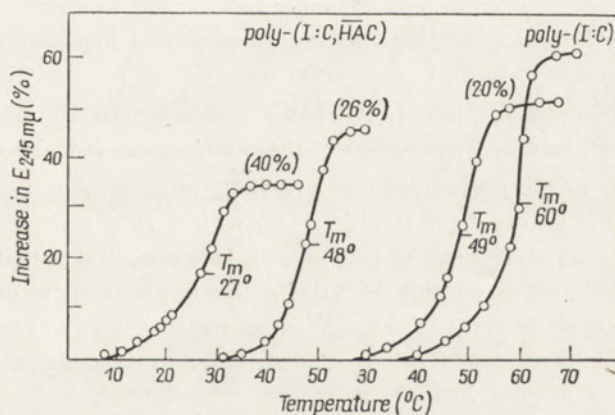

Fig. 3. Thermal transition profiles, in 0.01 M-phosphate buffer, pH 7.0, and 0.1 M-NaCl, of poly--(I:C) and poly-(I:C,$\overline{HAC}$). The figures in brackets indicate the $\overline{HAC}$ contents of the various copolymers. Measurements were made at the 245 mμ maximum (see Fig. 4). For the complex with the copolymer containing 40% $\overline{HAC}$ residues, the increase in extinction with temperature was measured relative to that at 2°; for the other complexes the increase was measured relative to that at 25°.
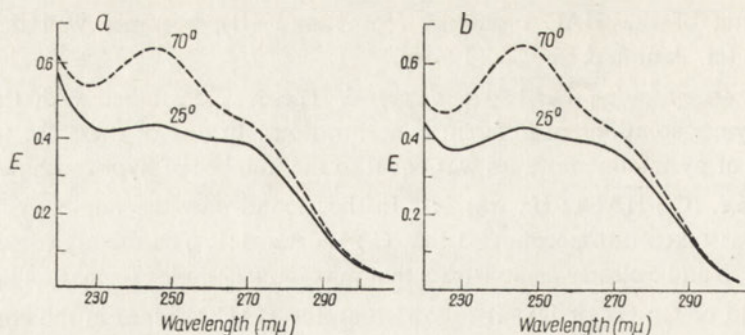
Fig. 4. Absorption spectra, in 0.01 M-phosphate buffer and 0.1 M-NaCl, of the twin-stranded complexes poly-(I:C,$\overline{HAC}$) at 25° (helical form) and 70° (melted, random coils): (*a*), for complex with copolymer containing 26% $\overline{HAC}$ residues; (*b*), for complex with copolymer containing 20% $\overline{HAC}$ residues.

The same figure exhibits the profile for poly-(I:C) for comparison purposes. For two of the complexes the absorption spectra for the helical and random coil forms are presented in Fig. 4. Attention is once more drawn to the fact that the differences in temperature hyperchromicity of the various complexes are due in part to the presence of non-complementary bases. This makes it all the more surprising that the breadths of the melting profiles are not appreciably increased as a result of the introduction of the non-complementary $\overline{HAC}$ residues. Fresco & Alberts (1960) observed appreciable broadening of the temperature profile (from 5° to 46°) for the twin-stranded poly-(U:A,U) in which the poly-A,U copolymer component contained 47% of U residues. Similar broad melting profiles were found by Szer & Shugar (1961) for poly-(A:U,3MeU) in which the 3MeU content of poly-(U,3MeU) was 33%. It is unlikely that these differences in breadths of melting profiles are due to stronger hydrogen bonding between cytosine and hypoxanthine base-pairs (as compared to adenine-uracil base pairing) since similar relatively narrow melting profiles were observed for poly-(A:U,$\overline{HAC}$) complexes to be described below. It also appears unlikely that the source of these differences is to be found in some special properties of the non-complementary $\overline{HAC}$ residues. An analoguos narrow temperature profile is found for poly-(A:U,C) with a 16% content of C residues in the poly-(U,C) copolymer strand (see Fig. 8). It is conceivable that the observed differences in behaviour may result from differences in chain length of the polymers utilized by various observers, or from differences in the degree of randomness with which the non-complementary residues are distributed in the copolymers. Relatively sharp temperature profiles have been recorded by Tsuboi, Matsuo & Nakanishi (1968) for poly-(I:C,U) complexes in which the U content of the poly-(U,C) was as high as 33.5% and 57%.

It was first demonstrated by Lipsett, Heppel & Bradley (1960, 1961) and subsequently by Michelson & Monny (1967), that the T_m and profile breadth of a given complex are dependent on the chain lengths of the polynucleotides employed. Maximum T_m values were observed for poly-(I:C) when the C chain contained
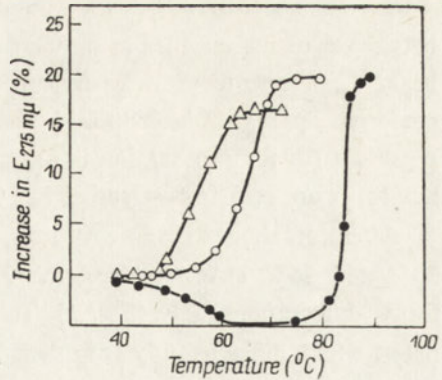
Fig. 5. Thermal transition profiles, in 0.01 M--acetate buffer, pH 4.05, and 0.1 M-NaCl, of poly-C (●); poly-(C,$\overline{\text{HAC}}$) containing 20% $\overline{\text{HAC}}$ residues (○); poly-(C,$\overline{\text{HAC}}$) containing 26% $\overline{\text{HAC}}$ residues (△). Measurements were made at 275 mμ and the ordinate scale gives the increase in extinction relative to that at 25°.

at least 12 residues, and for poly-(A:2U) when the number of U residues in the poly-U chain was at least 16. A copolymer containing non-complementary base residues may be regarded as a chain in which the sequences of complementary residues are "fragmented" by the former. The resulting complex of the copolymer with a homopolymer may then be likened to that of a complex between a long homopolymer chain and short complementary oligonucleotides, with this difference that the linkage of the short fragments by the non-complementary residues may facilitate complexing to the long chain and, perhaps, even give a more stable complex than would be obtained with isolated short fragments.

Consequently the T_m and profile breadth for a complex between a homopolymer and copolymer will depend on the relative stretches of complementary residues in the copolymer. The absence of any strict correlation between the percentage content of non-complementary bases, and the value of T_m, is undoubtedly due to the degree of "fragmentation" of the poly-(C,$\overline{\text{HAC}}$) chain by $\overline{\text{HAC}}$ residues.

"Acid" form of poly-(C,$\overline{\text{HAC}}$). In acid medium poly-C is known to form a twin--stranded helix, the properties of which have been described by a number of observers (Langridge & Rich, 1963; Akinrimisi, Sander & Ts'O, 1963; Hartman & Rich, 1965; Guschlbauer, 1967). The absorption of poly-(C,$\overline{\text{HAC}}$) at pH 4.05 was therefore examined as a function of temperature. A comparison of melting profiles between poly-C and poly-(C,$\overline{\text{HAC}}$) (Fig. 5) demonstrated that the latter does form a "helical" structure in acid medium, the T_m of which is appreciably decreased by the presence of $\overline{\text{HAC}}$ residues. Since the formation of acid poly-C is dependent on the protonation of cytosine "base-pairs", and hence on the pK for protonation of the ring $N_{(3)}$ nitrogen of C residues, the decrease in T_m of the acid form of the copolymer could conceivably be due to the lower pK of $\overline{\text{HAC}}$ (2.8, Janion & Shugar, 1965b) as compared to C (4.45, Shugar & Fox, 1952), if we assume that the $\overline{\text{HAC}}$ residues are in the amino form. However, Brown, Hewlins & Schell (1968) have recently provided evidence for the existence of $\overline{\text{HAC}}$ predominantly in the oximino form, $=N^4OH$, which

if true (see Discussion, below), would not allow for the type of base-pairing found between cytosine residues in the acid form of poly-C. Even if we assume $\overline{HAC}$ to be in the amino form, there remain doubts as to whether the protonated species could base pair with itself; poly-N^4-methylcytidylic acid, the base residues of which possess a pK for protonation (4.4) only slightly lower than that for cytosine, does not form an acid twin-stranded helix (Brimacombe & Reese, 1966).

Attention should be drawn to the fact that, for the two copolymers containing 20% and 26% $\overline{HAC}$ residues, the T_m values and hyperchromicities of the acid forms differ appreciably (Fig. 5). The same two copolymers give complexes with poly-I which differ by only 1° in their T_m values, although that with the lower $\overline{HAC}$ content exhibits, as might be expected, higher temperature hyperchromicity. The larger effect of an increase in the $\overline{HAC}$ content on the structure of the acid forms is probably due to the required closer packing of cytosine residues in the latter due to the presence of a third "ionic" bond, and the steric hindrance to such packing by the presence of additional $\overline{HAC}$ residues.

Bearing in mind that the pK of the $\overline{HAC}$ residues is in the neighbourhood of 2.8, attempts were made to examine the properties of the acid forms of the copolymers in the neighbourhood of pH 3. However, these attempts were unsuccessful due to precipitation of the copolymers, during heating, at pH values below 3.8.

Copolymers of U and $\overline{HAC}$, poly-(U,$\overline{HAC}$). Two such preparations were obtained, with $\overline{HAC}$ contents of 24% and 58%. The residual hyperchromicities of these, on hydrolysis to mononucleotides with pancreatic ribonuclease, were 7 and 11% respectively, as compared to 9% for poly-U itself. The absorption spectra of the latter two preparations, prior to and following hydrolysis to monomers, are presented in Fig. 6. Note in particular the *hypo*chromicity of both samples at wavelengths to the red of 290 mμ, indicative of some degree of base stacking in these polymers.

Complexes of poly-(U,$\overline{HAC}$) with poly-A. As for complexes of poly-(C,$\overline{HAC}$) with poly-I, those of poly-(U,$\overline{HAC}$) with poly-A were examined with the aid of solutions in which (a) the number of U residues was equal to the number of A residues, and (b) the number of U+$\overline{HAC}$ residues equalled the number of A residues.

For a $\overline{HAC}$ content of 24%, the mixing curves (Fig. 7) demonstrate unequivocally that the $\overline{HAC}$ residues do not participate in complex formation. When the $\overline{HAC}$ content is increased to 57%, no interaction of poly-(U,$\overline{HAC}$) is observed with poly-A even when the temperature is reduced to 2°. These results support our previous findings regarding the inability of homopolymers of $\overline{HAC}$ to complex with other potentially complementary homopolymers.

Melting profiles of poly-(U,$\overline{HAC}$) with poly-A. The thermal profiles of poly-(A:U) and poly-(A:U,$\overline{HAC}$) (with an $\overline{HAC}$ content for the latter of 24%) are shown in Fig. 8. For comparison purposes the same figure exhibits the helix-coil

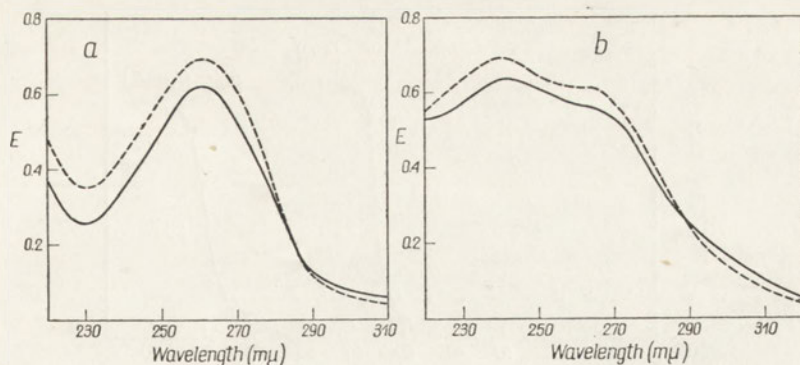Fig. 6. Absorption spectra of the copolymers poly-(U,$\overline{HAC}$) in 0.01 M-tris buffer, pH 7.8; (————) prior to, and (------) following hydrolysis to monomers with 20 μg./ml. pancreatic ribonuclease at 37° for 2 hr.: (*a*), copolymer containing 24% $\overline{HAC}$ residues; (*b*), copolymer containing 58% $\overline{HAC}$ residues.
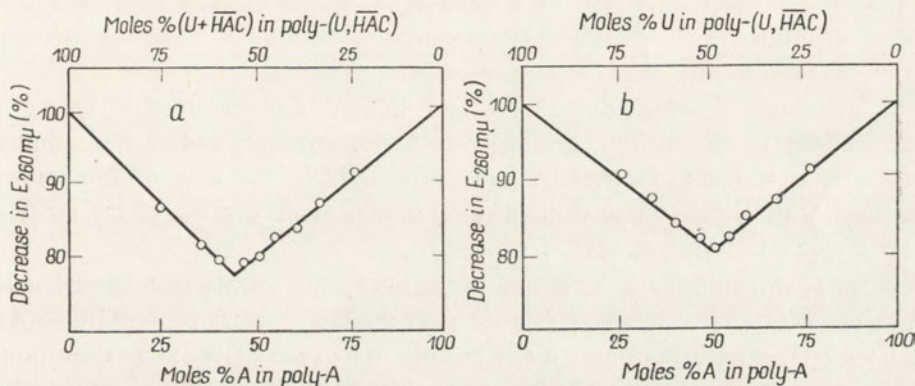

Fig. 7. Mixing curves, in 0.01 M-phosphate buffer, pH 7.0, and 0.1 M-NaCl of poly-A with poly--(U,$\overline{HAC}$) containing 24% $\overline{HAC}$ residues: (*a*), number of U+$\overline{HAC}$ residues taken equal to number of A residues; (*b*), number of U residues taken equal to number of A residues.

transition of poly-(A:U,C) in which the C content of the poly-(U,C) copolymer is 16%.

As in the case of the complexes between poly-I and poly-(C,$\overline{HAC}$) it can be seen from Fig. 8 that the presence of a non-complementary base residue in the copolymer component leads to a broadening of the thermal profile of the complex and a decrease in the T_m value. The results for the poly-(A:U,C) complex, where there is no doubt about the lack of complementarity between C and A, confirm the absence of complementarity between $\overline{HAC}$ and A.
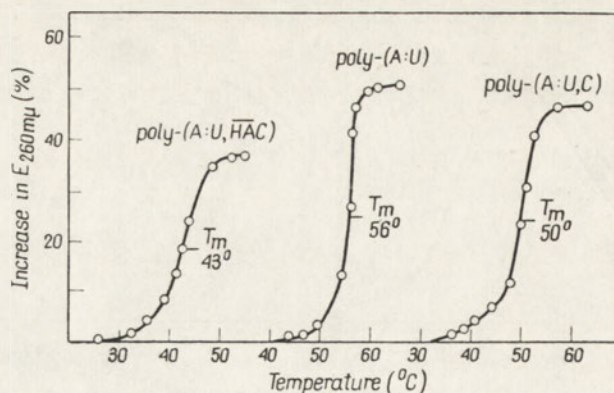
Fig. 8. Thermal transition profiles, in 0.01 M-phosphate buffer, pH 7.0, and 0.1 M-NaCl, of (from left to right) poly-(A:U,HAC) in which the copolymer component contained 24% HAC residues; poly-(A:U); and poly-(A:U,C) in which the poly-(U,C) contained 16% C residues. Measurements were at 260 mμ and ordinates give extinction increase relative to that at 25°.

DISCUSSION

Point mutations, both spontaneous and induced, are presumed to be due to modifications in base-pairing arising from tautomerization and/or ionization of specific base residues. Frequently, and particularly in the case of spontaneous mutations, this is difficult to demonstrate experimentally because of the rarity of these tautomeric forms at neutral pH.

From *in vivo* studies on the source of the mutagenic activity of hydroxylamine, which can generally be interpreted in terms of the base-pair transition G:C --> A:T (or C --> T(U) in a single strand), it was postulated by Freese (1963) that substitution of one of the exogenous amino hydrogens of cytosine by the strongly electronegative —OH group might lead to an increase in the tautomeric form with a hydrogen on the ring $N_{(3)}$ nitrogen, so that it would resemble uracil and base-pair like the latter to adenine. More recently Brown *et al.* (1968) claim, in fact, to have demonstrated that N^4-hydroxycytidine is largely in the oximino form, i.e. $=N^4OH$ (see Scheme 1, above), in aqueous medium at neutral pH. However, their conclusions, which are based on a comparison of the ultraviolet and infrared spectra of a series of model derivatives, are not as fully convincing as might be desired. In particular the infrared data, derived from solutions in non-aqueous solvents, do not necessarily reflect the situation existing in aqueous medium at neutral pH. While we are inclined to accept the conclusions of Brown *et al.* (1968) based on the I.R. data in non--aqueous medium, it is unfortunate that solubility considerations make it difficult to examine the infrared spectra in D_2O.

The results obtained in this study with copolymers, and in the previous study with homopolymers (Janion & Shugar, 1968), may be interpreted without any

assumptions as to the tautomeric form of the hydroxycytosine residues. Unquestionably those based on the use of copolymers inspire more confidence since they provide a closer approximation to natural conditions; and might also be expected to more readily demonstrate binding of hydroxycytosine residues when the association constant of these with another base is lower than that normally encountered (see next paragraph). For example, studies of the complexing properties with poly-I of copolymers of cytidylic acid and N^4-methylcytidylic acid (Brimacombe & Reese, 1966; Rabczenko & Szer, 1967) demonstrated that replacement of a hydrogen by a methyl in the exogenous amino group of cytosine led to a pronounced weakening, but not elimination, of base-pairing to hypoxanthine residues in poly-I. The failure of N^4-hydroxycytosine residues in both homo- and copolymers to associate with potentially complementary bases in other homopolymers appears to be unequivocal, and suggests that factors other than simple base-pairing are involved in the mutagenic process.

Particularly pertinent in this connection is a recent study by Hewlins (1967), who examined the association of hydroxycytidine with adenosine in appropriate non-aqueous medium (chloroform). Association was shown to occur *via* hydrogen bonding, but the association constant was relatively low, about 120, as compared to 550 for the A:U base pair, and 115 for the self-association of U residues. It is perhaps questionable whether such a low association constant could account for the specific binding of adenine to hydroxycytosine during the replication of a growing complementary chain without the intervention of other factors (see below).

The foregoing recalls the earlier finding, confirmed by Hewlins (1967), that dihydrouracil associates by means of hydrogen bonding with adenine in non-aqueous media, with an association constant about one-half that for the A:U pair (Kyogoku, Lord & Rich, 1966), whereas in aqueous medium poly-dihydrouridylic acid shows no ability to complex with poly-A (Cerutti, Miles & Frazier, 1966), if we accept the conclusion of the latter authors that the photochemical reduction of poly-U does not result in side reactions such as ring opening and/or chain scission.

Additional pertinent information is forthcoming from studies on *in vitro* template systems. As already referred to above, hydroxylamine-treated poly-C, when used as a matrix, directs the incorporation of adenine in place of guanine. When the matrix consists of a copolymer of C and N^4-hydroxy-C residues, the replacement of guanine by adenine in the newly synthesized complementary strand has been reported to be as high as 50% (Banks, Brown & Grossman, cited in Phillips & Brown, 1967), although full details regarding these results have apparently not yet been published. This is in obvious agreement with *in vivo* findings indicating that hydroxylamine induces G:C → A:T transitions. On the other hand, Budowsky, Sverdlov & Osterman (1967) report that when the matrix is natural DNA, N^4-hydroxycytidine-5′-triphosphate can efficiently replace CTP, but not UTP, in the newly formed chain, i.e. under these conditions C residues do not undergo transitions.

These apparently conflicting findings show that the behaviour of the same base, i.e. hydroxycytosine, depends on whether it forms part of the matrix or of the newly

synthesized chain. This bears some analogy to the behaviour of 5-bromouracil, which can replace only thymine during incorporation (Dunn & Smith, 1954); and following incorporation leads to an increase in the frequency of mutations (Litman & Pardee, 1956).

It is therefore apparent that hydroxylamine mutagenesis is more complex than appears at first sight and that, with the data currently available, cannot be interpreted uniquely in terms of modifications of base pairing. The findings with the RNA polymerase system suggest that the specificity of this enzyme may be at least partially involved.

REFERENCES

Akinrimisi E. O., Sander C. & Ts'O P.O.P. (1963). *Biochemistry* 2, 340.
Bautz-Freese E. & Freese E. (1964). *Proc. Nat. Acad. Sci. U. S.* 52, 1289.
Brimacombe R. L. C. & Reese C. B. (1966). *J. Mol. Biol.* 18, 529.
Brown D. M. & Hewlins M. J. E. (1968). *J. Chem. Soc.* p. 1922.
Brown D. M., Hewlins M. J. E. & Schell P. (1968). *J. Chem. Soc. (C)*p. 1925.
Brown D. M. & Phillips J. H. (1965). *J. Mol. Biol.* 11, 663.
Brown D. M. & Schell P. (1961). *J. Mol. Biol.* 3, 709.
Brown D. M. & Schell P. (1965). *J. Chem. Soc.* p. 208.
Budowsky E. I., Sverdlov E. D. & Osterman L. A. On *Symposium "Molekularnaya Biologia"*, *Moscow, 12-17 June, 1967*.
Cerutti P., Miles H. T. & Frazier J. (1966). *Biochem. Biophys. Res. Commun.* 22, 466.
Champe S. P. & Benzer S. (1962). *Proc. Nat. Acad. Sci. U. S.* 48, 532.
Dunn D. B. & Smith J. D. (1954). *Nature* 174, 305.
Fasman G. D., Lindblow C. & Grossman L. (1964). *Biochemistry* 3, 1015.
Freese E. (1959). *Proc. Nat. Acad. Sci. U. S.* 45, 622.
Freese E. (1963). In *Molecular Genetics* (J. H. Taylor, ed.) part I, p. 207. Academic Press, New York.
Freese E., Bautz E. & Bautz-Freese E. (1961a). *Proc. Nat. Acad. Sci. U. S.* 47, 845.
Freese E., Bautz-Freese E. & Bautz E. (1961b). *J. Mol. Biol.* 3, 133.
Freese E. & Strack H. B. (1962). *Proc. Nat. Acad. Sci. U. S.* 48, 1796.
Fresco J. R. & Alberts B. M. (1960). *Proc. Nat. Acad. Sci. U. S.* 46, 311.
Guschlbauer W. (1967). *Proc. Nat. Acad. Sci. U. S.* 57, 1441.
Hartman K. A. Jr. & Rich A. (1965). *J. Amer. Chem. Soc.* 87, 2033.
Hewlins M. J. E. (1967). *Ph. D. Thesis*, Cambridge University.
Janion C. & Shugar D. (1965a). *Biochem. Biophys. Res. Commun.* 18, 617.
Janion C. & Shugar D. (1965b). *Acta Biochim. Polon.* 12, 337.
Janion C. & Shugar D. (1968). *Acta Biochim. Polon.* 15, 107.
Kimhi Y. & Littauer U. Z. (1968). *J. Biol. Chem.* 243, 231.
Kochetkov N. K., Budowsky E. I., Sverdlov E. D., Shibaeva R. P., Shibaev V. N. & Monastyrskaya G. S. (1967). *Tetrahedron Letters* 34, 3253.

Kyogoku Y., Lord R. C. & Rich A. (1966). *Proc. Nat. Acad. Sci. U. S.* **57**, 250.

Langridge R. & Rich A. (1963). *Nature* **198**, 725.

Lawley P. D. (1967). *J. Mol. Biol.* **24**, 75.

Lipsett M. N., Heppel L. A. & Bradley D. F. (1960). *Biochim. Biophys. Acta* **41**, 175.

Lipsett M. N., Heppel L. A. & Bradley D. F. (1961). *J. Biol. Chem.* **236**, 857.

Litman R. M. & Pardee A. B. (1956). *Nature* **178**, 529.

Matthaei H., Heller G., Voigt H. P., Neth R., Schoch G. & Kubler H. (1967). In *Genetic Elements* (D. Shugar, ed.) p. 233. PWN and Academic Press, Warsaw and New York.

Michelson A. M. & Monny C. (1967). *Biochim. Biophys. Acta* **149**, 107.

Nagata C. & Mårtensson O. (1968). *J. Theoret. Biol.* **19**, 133.

Phillips J. H. & Brown D. M. (1967). *Progress in Nucleic Acid Research and Molecular Biology* **7**, 349. Academic Press Inc., New York.

Phillips J. H., Brown D. M., Adman R. & Grossman L. (1965). *J. Mol. Biol.* **12**, 816.

Phillips J. H., Brown D. M. & Grossman L. (1966). *J. Mol. Biol.* **21**, 405.

Rabczenko A. & Szer W. (1967). *Acta Biochim. Polon.* **14**, 369.

Schuster H. (1963). *Virology* **19**, 421.

Shugar D. & Fox J. J. (1952). *Biochim. Biophys. Acta* **9**, 199.

Szer W. & Shugar D. (1961). *Acta Biochim. Polon.* **8**, 235.

Tessman I., Poddar R. K. & Kumar S. (1964). *J. Mol. Biol.* **9**, 352.

Tsuboi M., Matsuo K. & Nakanishi M. (1968). *Biopolymers* **6**, 123.

Wilson R. C. & Caicuts M. J. (1966). *J. Mol. Biol.* **241**, 1725.

MECHANIZM MUTAGENICZNEGO DZIAŁANIA HYDROKSYLAMINY. WŁAŚCIWOŚCI KOMPLEKSOWANIA KOPOLIMERÓW KWASU HYDROKSYCYTYDYLOWEGO Z KWASEM CYTYDYLOWYM LUB URYDYLOWYM

Streszczenie

1. W reakcji enzymatycznej z fosforylazą polinukleotydową przygotowano dwie serie kopolimerów zawierających: a) mieszaninę reszt urydyny i N^4-hydroksycytozyny, b) mieszaninę reszt cytydyny i N^4-hydroksycytydyny. Opisano właściwości otrzymanych kopolimerów.

2. Z otrzymanych kopolimerów i z poli-A, lub poli-I, wytwarzano kompleksy i przebadano ich właściwości.

3. Stwierdzono, że reszty N^4-hydroksycytydyny w odpowiednich kopolimerach nie wytwarzają wiązań wodorowych z resztami adenozyny w poli-A, względnie z resztami inozyny w poli-I.

4. Kopolimery cytydyny i N^4-hydroksycytydyny są w dalszym ciągu zdolne do tworzenia dwuniciowej ,,kwaśnej" struktury podobnej do wytwarzanej w warunkach kwaśnych przez poli-C, ale bez udziału reszt N^4-hydroksycytozynowych.

5. Całość wyników jest zgodna z danymi uzyskanymi poprzednio przy użyciu homopolimerów, a mianowicie że reszty N^4-hydroksycytydyny nie tworzą wiązań wodorowych ani z adeniną, ani z inozyną.

6. Uważa się, że mutageneza działania hydroksylaminy nie polega na prostej tranzycji zasad, ale prawdopodobnie inne czynniki, jak np.: specyficzność enzymów replikujących, biorą udział w tym procesie.

RECENZJE KSIĄŻEK

PROCEEDINGS OF THE FOURTH MEETING OF THE FEDERATION OF EUROPEAN
BIOCHEMICAL SOCIETES, OSLO, 3-7 JULY 1967. Universitets forlaget, Oslo-Academic
Press, London, New York 1968; 6 tomów, łącznie str. 899, cena Ł 17.9.6

W ramach IV zjazdu Federacji Europejskich Towarzystw Biochemicznych odbywającym się
w Oslo w lipcu 1967 r. zorganizowano sześć całodziennych lub półdziennych sympozjów na następujące tematy (w nawiasach podano organizatorów poszczególnych sympozjów): 1. regulacja
aktywności enzymów i efekt allosteryczny (J. P. Changeux, Paryż), 2. biochemia replikacji wirusów
(A. P. Nygaard, Bergen), 3. struktura i funkcja tRNA i 5S-RNA (H. G. Zachau, Monachium),
4. wewnątrzkomórkowa lokalizacja i regulacja metabolizmu kwasów tłuszczowych (J. Bremer,
Oslo), 5. regulacja metabolizmu glikogenu (W. J. Whelan, Londyn) i 6. struktura i funkcja endo-
plazmatycznego retikulum (P. N. Campbell, Londyn). Pełny tekst 50 referatów wygłoszonych w ra-
mach tych sympozjów został wydany w formie sześciu oddzielnych tomików.

Nie sposób omówić tu choćby krótko treści tego interesującego wydawnictwa. Ograniczę się
więc do podkreślenia niektórych tylko, ciekawszych zagadnień w nim poruszonych.

Tom zatytułowany ,,Regulation of enzyme activity and allosteric interactions'' (redaktorzy
E. Kvamme i A. Pihl) dostarcza przede wszystkim nowych danych o regulacji aktywności enzymów
poprzez zmiany konformacji ich cząsteczki i o allosterycznym oddziaływaniu aktywatorów i inhi-
bitorów. Omówiono m.in. dehydrogenazę alkoholową, karbamoilotransferazę asparaginianową,
glutaminazę, fosforylazę.

Tom pt. ,,The biochemistry of virus replication'' (redaktorzy S. G. Laland i L. O. Fröholm)
omawia regulację syntezy RNA i białek w komórkach bakterii zakażonych bakteriofagiem oraz
mechanizmy transkrypcji i replikację wirusowego kwasu nukleinowego.

W tomie ,,Structure and function of transfer RNA and 5S-RNA'' (redaktorzy L. O. Fröholm
i S. G. Laland) zebrano referaty poświęcone tym tak ważnym i obecnie intensywnie badanym
,,niskocząsteczkowym'' kwasom nukleinowym. Omówione zostały prace nad strukturą tych związków
oraz ich oddziaływaniem z enzymami i rybosomami.

Sympozjum IV i poświęcony mu tom pt. ,,Cellular compartmentalization and control of
fatty acid metabolism'' (redaktor F. C. Gran) koncentruje się zasadniczo na trzech problemach:
a) biosyntezy kwasów tłuszczowych, b) roli karnityny w metabolizmie kwasów tłuszczowych i c) roli
fosfolipidów w komórce, a w szczególności ich roli w tworzeniu błon biologicznych.

Tom pt. ,,Control of glycogen metabolism'' (redaktor W. J. Whelan) jest odbiciem szybkiego
postępu osiągniętego w ostatnich latach w badaniach nad syntezą i rozpadem glikogenu w ko-
mórce zwierzęcej. Omawia on m.in. różne mechanizmy (hormonalne i niehormonalne) regulujące
syntezę glikogenu w wątrobie, metabolizm glikogenu w innych tkankach (m.in. zmiany enzyma-
tyczne towarzyszące patologicznym odchyleniom w metabolizmie glikogenu w leukocytach), pierwsze
etapy zamiany glikogenu w glukozę. Przedstawiono również badania nad ultrastrukturą ziarn gli-
kogenu przy pomocy mikroskopu elektronowego. Technika ta pozwoliła również zaobserwować
syntezę glikogenu pod wpływem fosforylazy.

Wreszcie tom zatytułowany ,,Structure and function of endoplasmic reticulum in animal cells''
(redaktor F. C. Gran) omawia kilka aspektów dotyczących roli endoplazmatycznego retikulum,
m.in. syntezę białka oraz procesy hydroksylacji.

W sumie omawiane wydawnictwo stanowi niezwykle cenny i bardzo ciekawy przegląd naj-
bardziej aktualnych zagadnień w obrębie sześciu wybranych problemów współczesnej biochemii.

Można żałować, że nie opublikowano dyskusji, jakie toczyły się po każdym referacie. Dyskusje te, często bardzo żywe, a czasem nawet zaciekłe, wniosły również wiele nowego do omawianych zagadnień. Trzeba jednak przyznać, że o ile rejestrowanie dyskusji w przypadku sympozjów zamkniętych o ograniczonej liczbie uczestników jest już bardzo trudne, to przy sympozjach otwartych i to o licznym, a czasem nawet tłumnym audytorium, byłoby chyba prawie niemożliwe.

Wszystkie tomiki są niezwykle starannie wydane, zaopatrzone w szczegółowe indeksy rzeczowe i wydrukowane na pięknym papierze.

Lech Wojtczak

BIOGENESIS OF NATURAL COMPOUNDS (P. Bernfeld, ed.). Pergamon Press, New York 1967; wyd. II rozszerzone i poprawione: str. 1209, cena £ 17.10.0

Książka poświęcona jest omówieniu procesów biosyntetycznych w świecie drobnoustrojów, roślin i zwierząt. Omówiono również w osobnych rozdziałach procesy syntetyczne zachodzące podczas odtruwania tkanek oraz sposób powstawania i nagromadzania w atmosferze wielopierścieniowych węglowodorów rakotwórczych.

Książka obejmuje 19 rozdziałów opracowanych przez znanych specjalistów, które poświęcone są m.in. omówieniu mechanizmów biosyntezy aminokwasów i białek, puryn, nukleotydów i kwasów nukleinowych, lipidów, sterydów, węglowodanów, karotenowców, witamin, tanin, terpenów, ligniny, alkaloidów i kauczuku. Tekst ilustrowany jest licznymi rysunkami, schematami, podano liczne wzory i reakcje. Na końcu znajduje się obszerny indeks rzeczowy oraz indeks cytowanych nazwisk. Jest to już z kolei drugie poszerzone wydanie książki Bernfelda, a poszczególne rozdziały zostały na nowo przerobione i uaktualnione z uwagi na dynamiczny rozwój badań w tym kierunku w ostatnich latach.

Szczególnie szeroko i wyczerpująco są opracowane rozdziały o biosyntezie białek (T. Peters), lipidów (K. P. Strickland), węglowodanów (P. Bernfeld), o biosyntezie substancji izoprenoidowych w tkankach roślinnych (H. J. Nicholas) oraz o biosyntezie alkaloidów (E. Leete). W każdym rozdziale cytowanych jest od kilkudziesięciu do kilkuset pozycji piśmiennictwa zebranego do roku 1966 włącznie. Większość autorów poszczególnych rozdziałów opisuje nie tylko mechanizm powstawania różnych grup związków chemicznych, ale również podaje metody śledzenia tych przemian oraz ich udział w procesach metabolicznych komórki.

To obszerne zbiorowe opracowanie daje doskonały przegląd wysiłków współczesnej biochemii w zakresie mechanizmu zachodzących w naturze syntez zarówno prostych związków organicznych, jak aminokwasy, cukrowce, zasady purynowe i pirymidynowe, jak i niezwykle złożonych struktur biologicznych, tj. białek, kwasów nukleinowych, wielocukrowców, kauczuku i innych polimerów. Mimo konieczności uzupełnień już w momencie wydania tego typu opracowania, książka Bernfelda jest wyjątkowo pożytecznym studium dla biochemików i chemików organików interesujących się procesami biogenezy najrozmaitszych substancji.

Włodzimierz Ostrowski

BIOCHEMICAL FACTORS IN ALCOHOLISM (R. P. Maickel, ed.). Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sidney, Paris, Braunschweig 1967; str. XI + 256, cena £ 4.0.0.

Omawiana książka stanowi zbiór artykułów z różnych dziedzin badań nad alkoholizmem. Jakkolwiek większość poruszonych zagadnień dotyczy — zgodnie z informacją zawartą w tytule książki — biochemicznych aspektów alkoholizmu, to jednak znaleźć tam można również wielkie bogactwo informacji na temat morfologicznych zmian narządów w alkoholizmie, na temat szeroko pojętej „patologii alkoholizmu", a nawet na tematy socjologiczne i terapeutyczne.

W ogólnym wstępie Ruth Fox zapowiada, że ta wartościowa książka stawia więcej pytań aniżeli przynosi odpowiedzi; autorka stara się zdefiniować pojęcie ,,alkoholizmu", omawia warunki powstawania tej choroby i przedstawia krótko sposoby postępowania przy jej zwalczaniu. Książkę podzielono na cztery części noszące następujące tytuły: I. Wpływ etanolu na procesy biochemiczne; II. Metabolizm etanolu u ssaków; III. Patologia wywoływana alkoholem u człowieka i zwierząt; IV. Aspekty terapeutyczne. Wszystkie artykuły napisali autorzy aktywnie zaangażowani w badania nad różnymi problemami alkoholizmu. W pierwszej części, obok typowych badań nad wpływem alkoholu na różne procesy biochemiczne w tkankach zwierzęcych, przedstawiono i inne ciekawe problemy. Na przykład O. F. Forsander z Laboratorium Fińskiego Monopolu Alkoholowego w Helsinkach w artykule p.t. ,,Rola metabolizmu w spożyciu alkoholu" podaje ciekawe dane dotyczące swobodnego wyboru spożycia alkoholu przez różne gatunki zwierząt doświadczalnych. Te doświadczenia nasuwają pewne podejrzenia co do roli dziedziczności w preferencji spożycia alkoholu u zwierząt; nakazują one także zwrócenie baczniejszej uwagi na czynniki genetyczne w alkoholizmie u ludzi. Spośród wielu bardzo interesujących prac zawartych w I części książki warto zwrócić uwagę na artykuł H. Kalanta i Y. Israela pt. ,,Wpływ etanolu na aktywny transport kationów". Wydaje się on poruszać szereg problemów o zasadniczym znaczeniu dla mechanizmu farmakologicznego działania alkoholu.

Druga część książki poświęcona jest metabolizmowi etanolu, a trzecia patologii (głównie wątroby) spowodowanej etanolem. Obydwie zawierają szereg prac przedstawiających wiele ciekawych doświadczeń, oryginalnych ujęć i schematów. Szkoda, że nie znalazł się tam żaden artykuł o pośmiertnej kinetyce znikania etanolu z krwi i tkanek, co ma istotne znaczenie sądowo-lekarskie. Czwarta część książki składa się tylko z jednego artykułu, w całości poświęconego leczeniu i rehabilitacji alkoholików. Dopełnia on całości obrazu alkoholizmu jako wielorakiego zaburzenia — fizjologicznego, psychologicznego, socjalnego i ekonomicznego.

Książka zainteresuje więc każdego, kto zechce badać zjawisko alkoholizmu lub zebrać informacje na tematy związane z działaniem lub przemianą etanolu. Korzystanie z książki jako ze zbioru wielu różnorodnych informacji byłoby niewątpliwie łatwiejsze, gdyby znalazł się w niej alfabetyczny skorowidz rzeczowy. Mimo tego mankamentu omawiana książka spełni zapewne zamierzony cel zbliżenia specjalistów z różnych dziedzin zajmujących się problemem alkoholizmu.

Mariusz Żydowo