POLSKA AKADEMIA NAUK
KOMITET BIOCHEMICZNY I BIOFIZYCZNY

POLISH ACADEMY OF SCIENCES
COMMITTEE OF BIOCHEMISTRY AND BIOPHYSICS

ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. 15
No. 4

WARSZAWA 1968
PAŃSTWOWE WYDAWNICTWO NAUKOWE

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Nakład 1809+131 Ark. wyd. 6,5, ark. druk. 4,875+0,5
Papier druk. sat. kl. III, 80 g. B1
Oddano do składania 25.IV.1968. Podpisano do druku 24.VII.1968.
Druk ukończono w sierpniu 1968.

Zam. 365/68 N-38 Cena zł 25.—

Warszawska Drukarnia Naukowa — Warszawa, Śniadeckich 8

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ACTA BIOCHIMICA POLONICA
Vol. 15 1968 No. 4

J. ZAJĄC

PHOSPHORYLATION OF PROTEIN IN LIVER AND KIDNEY MITOCHONDRIA OF THE RAT

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1. Participation of substrate level and oxidative phosphorylations in the phosphorylation of acid-stable phosphoprotein in rat kidney mitochondria, has been demonstrated using inhibitors and 2,4-dinitrophenol. 2. Of the phosphate acceptors, ADP stimulated in rat liver and kidney mitochondria the phosphorylation of acid-stable phosphoproteins and inhibited the phosphorylation of acid-labile phosphoprotein; GDP inhibited the phosphorylation of both phosphoprotein fractions. AMP had a similar effect. 3. The proposed mechanism of phosphorylation of acid-stable phosphoprotein in mitochondria has been discussed.

Two kinds of phosphoproteins are present in mitochondria. On the basis of susceptibility of their phosphate residues toward acid and alkali, they have been called, respectively, acid-stable and acid-labile phosphoproteins (Wadkins, 1963). The phosphorylated form of succinyl-CoA synthetase (EC 6.2.1.4) which participates in substrate level phosphorylation (Mitchell, Butler & Boyer, 1964), is the best known, even if not the only one, representative of the acid-labile fraction. On the other hand, the role of the acid-stable fraction is not yet clear. ATP is known to be involved in the phosphorylation of this fraction (Kennedy & Smith, 1954; Bieber & Boyer, 1966). It has been suggested that in liver mitochondria the phosphorylation may occur with the participation of the intermediates of oxidative phosphorylation (Ahmed & Judah, 1963; Sperti, Pinna, Lorini, Moret & Siliprandi, 1964). This suggestion is open to doubt in the light of the recent studies of Bieber & Boyer (1966) who demonstrated that oligomycin strongly inhibits the labelling of P-Ser in the presence of β-hydroxybutyrate. Sperti *et al.* (1964) have reported on the participation of substrate level phosphorylation in the phosphorylation of acid-stable phosphoproteins in liver mitochondria, but this requires confirmation. It is also necessary to compare the phosphorylation of proteins in mitochondria of tissues differing both in their structure and function, as all the reports concerning this problem dealt mainly with liver mitochondria.

The aim of this work was to study whether substrate level phosphorylation is involved in the phosphorylation of acid-stable phosphoproteins in kidney mitochondria, and to determine the effect of phosphate acceptors on phosphorylation of phosphoproteins in liver and kidney mitochondria.

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MATERIALS AND METHODS

Mitochondria from rat liver and kidney were obtained according to Plummer (1965) except that 0.25 M-sucrose solution containing 0.05 M-tris-Cl^- buffer, pH 7.4, and 2.5 mM-EDTA was used. Aged mitochondria were obtained by incubation for 1.5 hr. at 30° without added substrate, or by cooling in an ice-bath for 6 hr.

Changes in the content of acid-labile phosphohistidine (P-His) protein and acid-stable phosphoserine (P-Ser) protein under different metabolic conditions were followed by incorporation of [^{32}P]orthophosphate. The standard incubation mixture contained: 60 mM-tris-Cl^- buffer, pH 7.4; 50 mM-sucrose, 50 mM-KCl, 5 mM-$MgCl_2$, 0.5 mM-EDTA, 10 mM-potassium 2-oxoglutarate; the final volume of the sample was 2 ml. After 1.5 min. at 30°, the ^{32}P-labelled orthophosphate was added and the mixture incubated for 4 min., if not otherwise indicated.

The labelling of phosphohistidine was assayed according to Bieber, Lindberg, Duffy & Boyer (1964) except that the trichloroacetic acid sediment, prior to being suspended in urea-NH_4OH solution, was washed with 0.06 M-trichloroacetic acid and water to remove any remaining acid-soluble compounds; then the original procedure was followed.

It was found that, after the protein had been submitted to acid hydrolysis in order to liberate orthophosphate from P-His, a more acid-stable and alkali-labile ^{32}P-labelled phosphoprotein remained in the sediment. In this sediment, after acid hydrolysis, the presence of [^{32}P]phosphoserine was demonstrated by ion-exchange and paper chromatography procedures and by electrophoresis, using a standard phosphoserine preparation. Therefore, after determination of P-His by the above method of Bieber *et al.* (1964), the protein sediment was dissolved in 85% formic acid and the radioactivity of P-Ser determined.

For radioactivity assay, a BAH type GM counter, with window weight of 4 mg./cm.^{2}, was used.

Protein was determined by the biuret method of Jacobs, Jacob, Sanadi & Bradley (1956).

Reagents: Potassium arsenite was prepared by dissolving As_2O_3 in a KOH solution; phosphoserine was prepared according to Neuhaus & Korkes (1958), yeast hexokinase was from Mann Research Lab. Inc. (New York, U.S.A.), CoA from L. Light (Colnbrook, England), AMP from Nutr. Biochem. Co. (Cleveland, Ohio, U.S.A.), ADP from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), GDP was a kind gift of Prof. Dr. O. Lindberg (Wenner-Gren Inst., Stockholm, Sweden), NAD was from C. F. Boehringer & Soehne, G.M.B.H. (Mannheim, Germany) and [^{32}P]orthophosphate was from the Institute for Nuclear Research (Warszawa, Poland).

RESULTS

Kidney mitochondria. The results of [^{32}P]orthophosphate incorporation into mitochondrial phosphoproteins in the presence of 2-oxoglutarate, some inhibitors and 2,4-dinitrophenol (DNP), are presented in Table 1. 2-Oxoglutarate stimulated

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Table 1

The effect of inhibitors and 2,4-dinitrophenol on the incorporation of [^{32}P]*orthophosphate into P-His and P-Ser of kidney mitochondria*

The incubation mixture contained: 60 mM-tris-Cl$^-$ buffer, pH 7.4; 50 mM-sucrose, 50 mM-KCl, 5 mM-$MgCl_2$, 0.5 mM-EDTA, 10 mM-potassium 2-oxoglutarate and other components as indicated in the Table; the final volume of the sample was 2 ml., and temperature of incubation 30°. Prior to the experiment, the mitochondria were kept for 1 - 1.5 hr. in an ice bath. After addition of mitochondria (11.6 mg./sample) the sample was incubated for 1.5 min., then [^{32}P]orthophosphate (0.08 μmole/sample, specific activity 2.51×10^8 counts/min./μmole) was added. After a further 4 min., the reaction was stopped by adding 8 ml. of cold 0.3 M-trichloroacetic acid containing 3 mM-KH_2PO_4.

Inhibitor	Activity (counts/min./mg. protein)			
	P-His		P-Ser	
	without DNP	with 0.1 mM-DNP	without DNP	with 0.1 mM-DNP
None, and 2-oxoglutarate omitted	531	455	228	247
None	778	508	854	589
Potassium malonate (10 mM)	320	320	304	474
Potassium maleate (10 mM)	891	683	589	474
Potassium arsenite (1 mM)	391	511	246	910
Sodium arsenate (5 mM)	210	228	380	228

Table 2

The effect of cofactors and phosphate acceptors on the incorporation of [^{32}P]*orthophosphate into P-His and P-Ser in kidney mitochondria*

Before the experiment, the mitochondria were kept for 3.5 hr. in an ice-bath. The composition of the incubation mixture and conditions of incubation were as described in Table 1 except that 17.6 mg. of protein/sample was taken. The [^{32}P]orthophosphate added (0.08 μmole/sample) had a specific activity of 2.54×10^8 counts/min./μmole. NAD and CoA were added to all proper samples, as indicated by arrow.

Additions	Activity (counts/min./mg. protein)	
	P-His	P-Ser
None (control)	660	548
NAD (0.5 mM), CoA (0.1 mM)	450	375
↓ GDP (0.2 mM)	325	250
↓ GDP (0.2 mM), ADP (0.5 mM)	138	126
↓ GTP (0.1 mM), ADP (0.5 mM)	138	126
↓ ADP (0.5 mM)	462	662

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Table 3

The effect of cofactors and phosphate acceptors on the incorporation of [^{32}P]*orthophosphate into P-His and P-Ser of liver mitochondria*

Before the experiment, the mitochondria were kept for 6 hr. in an ice-bath. The composition of the incubation mixture and conditions of incubation were as described in Table 1 except that 16.8 mg. of protein/sample was taken. The [^{32}P]orthophosphate added (0.08 μmole/sample) had a specific activity of 3×10^8 counts/min./μmole.

Additions	Activity (counts/min./mg. protein)	
	P-His	P-Ser
None (control)	807	631
NAD (0.5 mM)	288	245
CoA (0.1 mM)	920	670
GDP (0.2 mM)	274	301
GDP (0.2 mM), ADP (0.5 mM)	315	480
ADP (0.5 mM)	1027	1192

Table 4

The effect of phosphate acceptors, GTP and NAD on the incorporation of [^{32}P]*orthophosphate into P-His and P-Ser in aged liver mitochondria*

The mitochondria, suspended in 0.25 M-sucrose containing 0.05 M-tris-Cl$^-$ buffer, pH 7.4, and 2.5 mM-EDTA, were kept for 1.5 hr. at 30° without the addition of substrate; then they were added to the incubation mixture described in Table 1 and incubated at 25°. The amount of mitochondria was 17.6 mg. of protein/sample and of [^{32}P]orthophosphate 0.25 μmole/sample (specific activity 5×10^7 counts/min./μmole).

Additions	Activity (counts/min./mg. protein)	
	P-His	P-Ser
None, and with 2-oxoglutarate omitted	81	86
None	148	287
GTP (0.05 mM)	45	62
GTP (0.05 mM), ADP (0.75 mM)	37	88
GTP (0.05 mM), AMP (1.00 mM)	70	148
ADP (0.75 mM)	135	370
AMP (1.00 mM)	41	67
NAD (0.50 mM)	60	99

the labelling of P-Ser almost fourfold. In the presence of 2-oxoglutarate, the incorporation of $^{32}P_i$ into both phosphoprotein fractions was inhibited by arsenate, arsenite and malonate, whereas maleate stimulated the labelling of P-His but inhibited that of P-Ser. DNP in the presence of 2-oxoglutarate inhibited the labelling of both phosphoprotein fractions only by about 30%. It enhanced the inhibition by arsenate of the labelling of P-Ser, whereas it abolished the inhibition by arsenite of the labelling of P-His and distinctly stimulated that of P-Ser.

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Of the phosphate acceptors listed in Table 2, only ADP stimulated the incorporation of $^{32}P_i$ into P-Ser, whereas GDP, especially in the presence of ADP, distinctly reduced the radioactivity of both phosphoprotein fractions. NAD and CoA added together inhibited partially the labelling of P-His and P-Ser.

Liver mitochondria. The "hexokinase trap" in the presence of 2-oxoglutarate rapidly and distinctly lowered the labelling of P-His, and had no effect on P-Ser (Fig. 1).

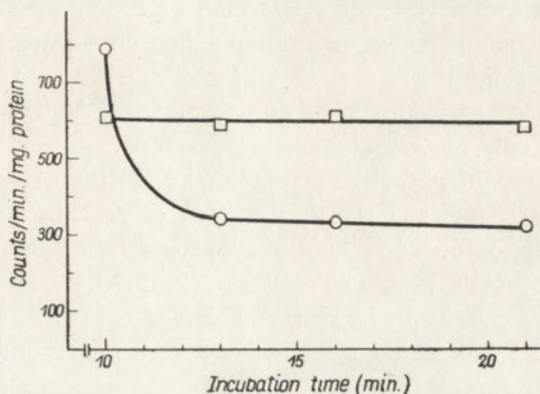

Fig. 1. The effect of hexokinase, ADP and glucose (the "hexokinase trap") on the ^{32}P-labelled phosphoprotein fractions of liver mitochondria. The mitochondria, prior to the experiment, were kept in an ice-bath for 1.5 hr., then added (90 mg. of protein) to the incubation mixture described in Methods; final volume 10 ml. After 1.5 min., [^{32}P]orthophosphate (0.4 μmole, specific activity 8.81×10^7 counts/min./μmole) was added. After a further 10 min., a 2 ml. portion was taken for determination of the label of P-His and P-Ser, and to the remaining mixture hexokinase (0.4 mg./ml.), 1 mM-ADP and 24 mM-glucose were added, and the incubation continued. At time intervals indicated in the Figure, samples were withdrawn for determinations of (○), P-His and (□), P-Ser.

The effect of cofactors and phosphate acceptors was assayed on aged mitochondria (Tables 3 and 4). The labelling of both phosphoprotein fractions was inhibited by GDP, AMP and GTP, whereas CoA abolished the inhibitory effect of NAD, and only ADP stimulated the incorporation of $^{32}P_i$ into P-Ser.

DISCUSSION

To study the role played by substrate level and oxidative phosphorylations in phosphorylation of protein, freshly prepared kidney mitochondria were used. The obtained results (Table 1) indicate the participation of both these processes. Pronounced stimulation by 2-oxoglutarate of P-Ser labelling, the extent of inhibition in the presence of 2-oxoglutarate by DNP, arsenite and arsenate, and arsenate and DNP added together, suggest the participation of substrate level phosphorylation. It should be added that maleate, which is known to inhibit specifically the oxidation of 2-oxoglutarate in kidney mitochondria (Angielski & Rogulski,

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1962), inhibited the labelling of P-Ser by 30 - 50% in kidney, but not in liver mitochondria (unpublished experiments). On the other hand, there is also evidence for the participation of oxidative phosphorylation in the phosphorylation of P-Ser; malonate inhibited the labelling of P-Ser, probably by blocking the citrate cycle at the step of succinate dehydrogenase and additionally by shifting the equilibrium of the reaction catalysed by succinyl-CoA synthetase. DNP, even in the presence of 2-oxoglutarate, inhibited in part the incorporation into P-Ser. Arsenate inhibited to a greater extent the labelling of P-His than of P-Ser, most probably because it has a weaker uncoupling effect on oxidative than on substrate level phosphorylation (Heldt, Jacobs & Klingenberg, 1964). Maleate, when used at a concentration which completely inhibits oxidation of 2-oxoglutarate, inhibited the incorporation of $^{32}P_i$ into P-Ser only by 30%.

In the experiments with phosphate acceptors, the labelling of P-Ser was stimulated by ADP. This clearly indicates the participation of ATP in the phosphorylation of acid-stable phosphoproteins in kidney mitochondria, all the more so that the presence of ATP : protein phosphotransferase (EC 2.7.1.37) in mitochondria has been demonstrated by Jackson, Jackson & Freeman (1965). Added GDP lowered the radioactivity of P-His in kidney mitochondria, probably due to the fact that it is an acceptor of phosphate from P-His, as it has been demonstrated for liver mitochondria (Heldt *et al.*, 1964; Slater & Kemp, 1964; Cha, Cha & Parks, 1965). Further studies are required to elucidate the observed similar effect of GDP on P-Ser.

To gain better knowledge of the mechanism of phosphorylation of the phosphoserine fraction with the participation of substrate level phosphorylation, the effect of phosphate acceptors on the labelling of phosphoprotein fractions in liver mitochondria has been assayed. The "hexokinase trap" in the presence of 2-oxoglutarate acted as an acceptor of phosphate from P-His (Fig. 1), and had no effect on P-Ser. Sperti *et al.* (1964) in a similar experiment but in the presence of β-hydroxybutyrate and oligomycin, have observed a decrease of P-Ser labelling, and an almost complete inhibition was observed (Bieber & Boyer, 1966) when the phosphate acceptor was added at the beginning of incubation. All these experiments indicate that substrate level phosphorylation, even during high utilization of ATP, provides a sufficient amount of the phosphate donor for P-Ser phosphorylation. However, it is not known whether this is P-His, GTP or ATP.

In the next experiments (Tables 3 and 4), aged mitochondria were used to eliminate the oxidative phosphorylation and to facilitate the penetration of the added cofactors. GDP, in agreement with the results of Lindberg, Duffy, Norman & Boyer (1965), lowered the labelling of P-His due to GDP being directly phosphorylated by P-His (Heldt *et al.*, 1964; Slater & Kemp, 1964; Cha *et al.*, 1965). On the other hand, the inhibitory effect of GDP on the labelling of P-Ser, observed in the present work, could indicate the direct phosphorylation of P-Ser by P-His; this conclusion, however, does not agree with the results obtained in the "hexokinase trap" experiment. Also Bieber & Boyer (1966) excluded the interaction of these two phosphoproteins.

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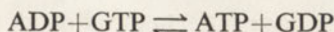
The inhibitory effect of GDP on the labelling of P-Ser seems to exclude also the direct participation of GTP in the phosphorylation of this protein. However, it is also possible that GDP may inhibit the utilization of GTP in mitochondria, directly or through ATP. In the latter case, the stimulation by ADP and the inhibition by GDP and "cold" GTP of the labelling of P-Ser could be explained by the reaction:

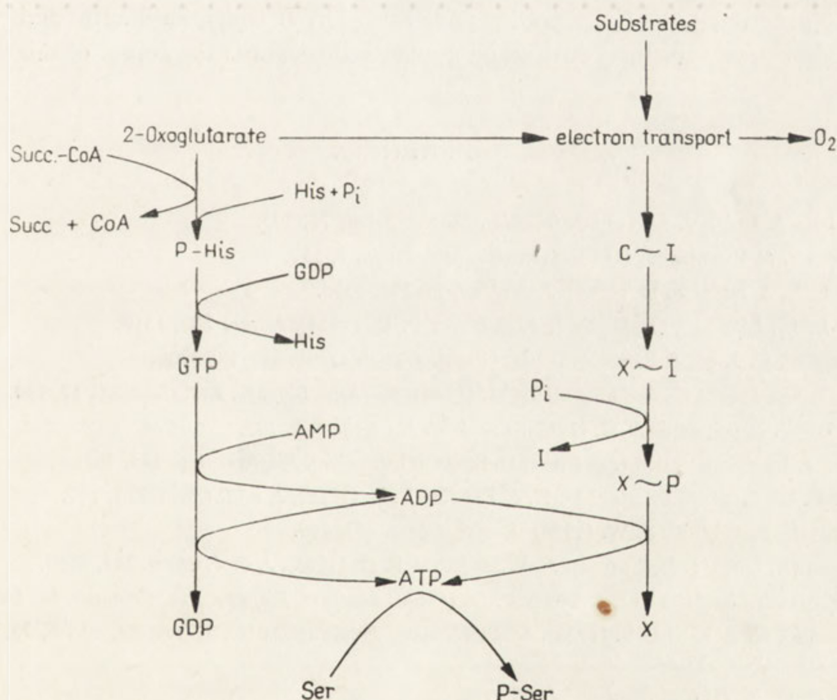


$$ADP+GTP \rightleftharpoons ATP+GDP$$

On the other hand, the inhibitory effect of AMP on the labelling of P-Ser could be explained by utilization of GTP in the phosphorylation of AMP to ADP. This possibility is supported by partial overcoming of the AMP effect by simultaneous addition of "cold" GTP. Such an explanation is in agreement with the report of Heldt & Schwalbach (1967) who have demonstrated the presence of GTP : AMP phosphotransferase (EC 2.7.4.10) in liver mitochondria.

Thus the results discussed above indicate that ATP is the direct phosphate donor also in substrate level phosphorylation of P-Ser.

On the basis of the results obtained both by other workers and in the present work, a mechanism of protein phosphorylation in kidney and liver mitochondria is proposed (Scheme 1). ATP is shown as the immediate precursor because only



Scheme 1. The proposed mechanism of protein phosphorylation in liver and kidney mitochondria. C ~ I, X ~ I and X ~ P, intermediates of oxidative phosphorylation; P-His and His, two forms of succinyl-CoA synthetase; P-Ser and Ser, phosphorylated and non-phosphorylated forms of acid-stable protein. Succ., succinate; succ.-CoA, succinyl-CoA.

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its participation in the phosphorylation of the acid-stable phosphoproteins has been sufficiently substantiated.

In the experiments in which the effect of phosphate acceptors was studied, two cofactors of 2-oxoglutarate oxidation, NAD and CoA, were also included, and NAD was found to inhibit the labelling of both phosphoprotein fractions. This is at variance with the results of Lindberg *et al.* (1965) showing that NAD stimulated the labelling of P-His in aged liver mitochondria obtained by shaking in phosphate buffer prior to washing with 0.25 M-sucrose. However, this procedure could have resulted in greater damage being incurred by mitochondria, and this might have brought about increased penetration of NAD and stimulation of 2-oxoglutarate oxidation, thus enhancing the labelling of P-His. The elucidation why NAD inhibits the labelling of both phosphoprotein fractions in aged undamaged mitochondria, and CoA abolishes this effect, requires further study.

More experiments are also necessary to elucidate why DNP overcomes the arsenite-induced inhibition of the labelling of phosphoprotein fractions in kidney mitochondria.

The author wishes to thank Prof. Dr. O. Lindberg of the Wenner-Gren Institute for the generous gift of the GDP; and Prof. Dr. J. Opieńska-Blauth and Doc. Dr. T. Borkowski for their advice and guidance throughout the course of this work.

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FOSFORYLACJA BIAŁEK W MITOCHONDRIACH WĄTROBY I NERKI SZCZURA

Streszczenie

1. Przy użyciu inhibitorów i 2,4-dwunitrofenolu wykazano udział fosforylacji substratowej i oksydacyjnej w fosforylacji fosfoproteidów kwasostabilnych w mitochondriach nerki szczura.

2. Spośród akceptorów fosforanu ADP stymuluje fosforylację fosfoproteidów kwasostabilnych, a hamuje fosforylację fosfoproteidów kwasolabilnych w mitochondriach wątroby i nerki szczura; GDP hamuje fosforylację obydwu frakcji fosfoproteidowych. Podobnie działa AMP.

3. Przedyskutowano prawdopodobny mechanizm fosforylacji kwasostabilnych fosfoproteidów w mitochondriach.

Received 26 January, 1968.

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A C T A B I O C H I M I C A P O L O N I C A

Vol. 15 1968 No. 4

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PURIFICATION AND PROPERTIES OF DIHYDRO-OROTASE FROM PEA PLANTS

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1. Dihydro-orotase from homogenates of young pea plants was purified 320-fold, and the isolated preparation proved to be free of any related enzymic activity. 2. The purified enzyme catalysed the reversible, stoicheiometric, conversion of carbamoylaspartate to dihydro-orotate with the apparent equilibrium constant of approx. 0.8 at pH 6.0. The minimal K_m for carbamoylaspartate was 6.2 mM and for dihydro-orotate 1.5 mM. 3. The enzyme was relatively stable, required no soluble cofactors and showed high substrate specificity. 4. The molecular weight of the enzyme estimated by Sephadex G-200 gel filtration, was about 110 000.

The main path of pyrimidine nucleotide biosynthesis is a multi-step process in which orotic acid is a key intermediate. Most of the enzymes involved in orotate formation and utilization in micro-organisms and animal tissues (for reviews see Reichard, 1959, and Wang & Waygood, 1964), have been recently found also in higher plants (Neumann & Jones, 1962; Kapoor & Waygood, 1965; Wolcott & Ross, 1967; Wang, 1967). So far, only dihydro-orotase (L-4,5-dihydro-orotate amidohydrolase, EC 3.5.2.3), responsible for the conversion of CA[1] to DHOA, has not been obtained in a purified form. Merely a crude enzyme preparation containing in addition a related dihydropyrimidinase activity, has been isolated from pea seedlings (Mazuś & Buchowicz, 1966). Further experiments (Mazuś & Buchowicz, 1967) have shown that these activities differ in heat-stability. Attempts were therefore made to obtain purified dihydro-orotase and dihydropyrimidinase, free of each other. This report describes isolation and some general properties of dihydro-orotase from pea plants. Isolation of dihydropyrimidinase is presented in the accompanying paper (Mazuś & Buchowicz, 1968).

[1] Abbreviations: CA, *N*-carbamoyl-L-aspartate; CBA, *N*-carbamoyl-β-alanine; CBAIB, *N*-carbamoyl-β-aminoisobutyrate; DHOA, L-4,5-dihydro-orotate; DHT, 4,5-dihydrothymine; DHU, 4,5-dihydrouracil.

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MATERIALS AND METHODS

Chemicals. DHOA, DHU, DHT, ox serum albumin and Aquacide I (Calbiochem., Los Angeles, Calif., U.S.A.); ATP and NAD (Sigma, St. Louis, Mo., U.S.A.); 2-mercaptoethanol (Koch and Light Lab., Colnbrook, Bucks., England); ox pancreatic RNase (Reanal, Budapest, Hungary); human fibrinogen (Behringewerke, Marburg, Germany); human γ-globulin (Biomed, Warsaw, Poland); CA, CBA, CBAIB, carbamoylglutamate and carbamoylglycine were synthesized according to the method of Nyc & Mitchell (1947); other reagents were from Fabryka Odczynników Chemicznych (Gliwice, Poland).

Plants. Top leaves of two-week-old green pea plants were used as starting material.

Enzyme assays. The forward (CA → DHOA) and backward (DHOA → CA) reactions require different pH values for maximal velocity (Mazuś & Buchowicz, 1966). Thus, two different incubation mixtures were used. Unless otherwise stated, these were prepared as follows. Assay system *a*, for the forward reaction: 0.1 ml. of 0.2 M-potassium phosphate - citrate buffer, pH 6.0, 1 μmole of the substrate, 1 μg. of the enzyme protein, and water to a final volume of 0.2 ml. Assay system *b*, for the backward reaction: 0.1 ml. of 0.2 M-potassium phosphate buffer, pH 8.0; substrate, enzyme and water in the same proportions as in system *a*.

In both cases the reaction was stopped by addition of 5 ml. of 0.3 N-$HClO_4$ after 1 hr. incubation at 37°. Identification of the products formed as well as quantitative changes in the substrate and product contents were determined according to methods quoted previously (Mazuś & Buchowicz, 1966).

Dihydropyrimidinase activity was determined under conditions described in the accompanying paper (Mazuś & Buchowicz, 1968).

Molecular weight determination. The approximate molecular weight of plant dihydro-orotase was estimated according to the method of Whitaker (1963). A Sephadex G-200 column (2 × 50 cm.) was calibrated with ox pancreatic RNase, ox serum albumin, human γ-globulin and human fibrinogen. The void volume of the column was established with Dextran blue 2000. About 100 μg. of the purified enzyme, dissolved in 0.5 ml. of 0.05 M-potassium phosphate buffer, pH 7.1, was applied on the top of the column previously equilibrated with the same buffer, and 0.5 ml. fractions were collected for enzyme activity determinations.

RESULTS

Enzyme purification

Crude preparation. The extract of top leaves of pea plants was prepared as previously described (Mazuś & Buchowicz, 1966), and the fraction precipitated at 0.4 - 0.65 ammonium sulphate saturation was collected. From 500 g. of fresh material about 1 g. of protein was obtained. The crude preparation contained dihydro-orotase and dihydropyrimidinase activities of the same magnitude.

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Heat treatment. In preliminary experiments a short heating was found to be adequate to destroy selectively dihydropyrimidinase (Table 1). Thus, 1 g. of the crude preparation was dissolved in 36 ml. of 0.1 M-potassium phosphate - citrate buffer, pH 6.0, and 4 ml. portions pipetted into test tubes. The samples were then placed in a hot water bath (70°) for 3 min., then immediately immersed in an ice-water mixture. All the subsequent operations were carried out in a cold-room at 4°. The samples were pooled and the protein precipitate formed on heating was discarded by low-speed centrifugation.

Table 1

Effect of heat treatment on catalytic activity of the crude dihydro-orotase preparation

About 220 mg. of protein (first ammonium sulphate precipitate) were dissolved in 50 ml. of 0.1 M-potassium phosphate - citrate buffer, pH 6.0; 4 ml. portions of the solution were immersed in a hot (70°) water bath for the indicated time. After cooling, the samples were centrifuged to remove protein precipitates formed on heating. The supernatants (20 µl. portions) were used for protein content and enzyme activity determinations (assay system *b*, see the Materials and Methods section). The results are expressed as µmoles of the substrate metabolized/mg. of protein/hr.

Time of heating at 70° (min.)	Protein concentration (mg./ml.)	Degradation of	
		dihydro-orotate	dihydro-uracil
0	4.50	1.10	1.10
1	1.80	1.71	1.06
3	1.13	2.09	0.00
5	1.10	1.75	0.00
7	1.04	1.00	0.00
9	1.04	0.00	0.00

Further purification. The supernatant after heat treatment (35 ml.) was again fractionated with ammonium sulphate, and the fraction precipitating at 0.2 - 0.4 saturation collected by centrifugation at 10 000 *g* for 10 min. The precipitate was dissolved in 2 ml. of 0.01 M-potassium phosphate buffer, pH 7.2, and applied to a Sephadex G-100 column (1 × 17 cm.), equilibrated with the same buffer. The effluent was collected in 1 ml. fractions at a flow rate of 0.5 ml./min. Dihydro-orotase activity was eluted between the 19th and 25th milliliter of the effluent. These fractions were pooled and layered onto a column of DEAE-cellulose (1 × 15 cm.), equilibrated with 0.01 M-potassium phosphate buffer, pH 7.2. The column was washed successively with 50 ml. portions of the above buffer, then with 50 ml. of 0.03 M-phosphate buffer of the same pH, and the effluents discarded. For enzyme elution, 0.05 M-phosphate buffer was used. Rate of flow was 0.5 ml./min. Most of dihydro-orotase emerged at elution volumes from the 6th to the 18th ml. These fractions were pooled and used for experiments. Some experiments were made on concentrated enzyme solution. For this purpose the DEAE column eluate (5 ml.)

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Table 2

Purification of dihydro-orotase from pea plants

Fresh top leaves, 500 g., were used for isolation of the enzyme (see the text for experimental details). For the activity determinations dihydro-orotate (DHOA) was used as substrate. The activity is expressed in μmoles of DHOA decomposed/mg. of protein/hr.

Purification step	Volume (ml.)	Protein (mg.)	Specific activity	Purification factor	Yield (%)
Extract	500	4350	0.7	1	100
Ppt. at 0.4 - 0.65 $(NH_4)_2SO_4$ sat.	36	1063	1.9	2.7	66.3
Heat treatment	35	150	3.3	4.7	16.3
Ppt. at 0.2 - 0.4 $(NH_4)_2SO_4$ sat.	2	27.5	15.2	22	13.7
Sephadex G-100	6	6.8	60	86	13.4
DEAE-cellulose	12	0.24	225	321	1.8

was placed in dialysis tubing and the bag covered with dry Aquacide I for 3 hr. at 4°. This procedure reduced the volume tenfold without apparent loss in dihydro-orotase activity.

The course of dihydro-orotase purification is presented in Table 2. It should be pointed out that the final activity varied from one preparation to another within ±50%. This was caused by different activity of the initial plant homogenates and some variability in the efficiency of the heating step. The yield could be considerably improved if the heat treatment was omitted but the final degree of purification was markedly lower, although in both cases the enzyme was free of dihydropyrimidinase activity. Sephadex gel filtration could be replaced by dialysis giving a preparation of similar final yield but of lower purity.

Enzyme properties

Stability. The plant dihydro-orotase proved to be relatively stable at all steps of purification. Considerable stability of the enzyme was first observed on heating of the crude preparation at 70° for 3 min. at pH 6.0 (Tables 1 and 2). The purified preparation in diluted solutions at pH 6.0 retained at 4° 50% of activity after four weeks. For the direct DEAE-cellulose column eluate (pH 7.2) the same loss of activity was observed after two weeks of storage. No loss of activity was observed when the enzyme solution was stored at -15° for a month.

Stoicheiometry and equilibrium. The stoicheiometry of the CA ⇄ DHOA interconversion was studied by analysing reaction mixtures after incubation. The results were consistent with the theoretical stoicheiometry within 5 - 10% error for the forward and backward reactions (Table 3). Incubation of 1 μmole of CA under conditions of assay system *a* (pH 6.0) for 1 hr. resulted in the disappearance of 0.24

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Table 3

Stoicheiometry and equilibrium of the dihydro-orotase-catalysed reaction

The purified preparation, 1 μg. of protein, was incubated with the substrates indicated at 37° either at pH 6.0 (assay system *a*) or at pH 8.0 (assay system *b*). The results are expressed as μmoles of carbamoyl-aspartate and dihydro-orotate found in the reaction mixtures at the end of the incubation period.

Substrates		Assay system	Incubation time (hr.)	Final content in the reaction mixture of	
carbamoyl-aspartate (μmole)	dihydro-orotate (μmole)			carbamoyl-aspartate (μmole)	dihydro-orotate (μmole)
1.00	0.00	*a*	1	0.76	0.22
1.00	0.00	*a*	2	0.64	0.34
1.00	0.00	*a*	3	0.62	0.38
1.00	0.00	*a*	4	0.58	0.40
0.55	0.45	*a*	1	0.55	0.49
0.00	1.00	*a*	1	0.18	0.83
0.00	1.00	*b*	1	0.56	0.50
1.00	0.00	*b*	1	0.95	0.06

Table 4

Effect of various additions to the incubation mixture on the dihydro-orotase-catalysed conversion of carbamoylaspartate to dihydro-orotate

The purified enzyme was incubated with CA and the compounds indicated under conditions of assay system *a* (see the Materials and Methods section). Enzyme tested without the additions catalysed formation of 230 μmoles of DHOA/mg. of protein/hr.

Additions	Concn. (mM)	Relative activity (%)	Additions	Concn. (mM)	Relative activity (%)
None	—	100	$HgCl_2$	1	0
$MgSO_4$	1	104	$AgNO_3$	1	0
$MnCl_2$	0.1	92	EDTA	1	86
$CoCl_2$	1	104	NAD	10	107
$ZnSO_4$	1	107	ATP	10	69
$NiCl_2$	1	85	2-Mercapto-ethanol	1	65
$CuSO_4$	1	71			

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μmole of the substrate and the appearance of 0.22 μmole of the product, i.e. DHOA. When the incubation time was extended to 2 hr., considerably more of CA was consumed and correspondingly more of DHOA was formed. Further prolongation of the incubation time led to only slight changes in composition of the reaction mixture. Thus, during the fourth hour of the incubation only 0.02 μmole of DHOA was formed. The observed decrease in the reaction rate resulted probably from approaching the equilibrium rather than from enzyme inactivation. To confirm this conclusion a reaction mixture of the composition found at the assumed equilibrium (about 55% CA and 45% DHOA) was prepared and incubated with the enzyme for an hour. No change in the amount of CA and only a slight increase of DHOA was observed. Thus, the apparent equilibrium constant for the conversion of CA to DHOA at pH 6.0 is approximately 0.8. At pH 8.0 (assay system *b*) the equilibrium was strongly shifted towards DHOA degradation, and cyclization of CA was barely detectable.

Cofactor requirement and inhibitors. The results of experiments showed (Table 4) that none of the divalent cations tested (Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+}) was essential for plant dihydro-orotase activity, and sodium versenate had no significant effect. In accordance with these results it was also observed that the enzyme withstood dialysis against water for one day. The product of the reaction, i.e. DHOA, was accumulated also in the presence of NAD, a cofactor required for enzymic oxidation of DHOA to orotate. ATP and 2-mercaptoethanol seemed to inhibit slightly the enzyme activity. Hg^{2+} and Ag^{1+}, but not Cu^{2+}, ions were strongly inhibitory.

Substrate specificity. The enzyme is highly specific as shown by its inability to catalyse interconversion of *N*-carbamoylamino acids and dihydropyrimidines other than CA and DHOA (Table 5). A slight decrease observed in the amounts of some of the substrates was never accompanied by formation of the expected product.

Table 5

Substrate specificity of plant dihydro-orotase

Purified enzyme was incubated with various substrates under the conditions of either assay system *a* or assay system *b* (see the Materials and Methods section), as indicated. The results are expressed as μmoles of the substrate consumed and product formed per sample.

Substrate	Assay system	Changes in the amount of	
		carbamoyl-compounds	dihydro-compounds
Carbamoylaspartate	*a*	—0.28	+0.26
Carbamoyl-β-alanine	*a*	—0.02	0.00
Carbamoyl-β-aminoisobutyrate	*a*	—0.01	0.00
Carbamoylglutamate	*a*	—0.01	0.00
Carbamoylglycine	*a*	0.00	0.00
Dihydro-orotate	*b*	+0.55	—0.53
Dihydrouracil	*b*	+0.01	0.00
Dihydrothymine	*b*	0.00	—0.03

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Influence of enzyme and substrate concentrations. A linear dependence of the rate of both dihydro-orotase-catalysed reactions and the enzyme concentration was observed in a range from 0.5 to 10 μg. of protein per 0.2 ml. of the standard incubation mixture. Higher concentrations of protein were not tested.

The K_m value for CA at pH 6.0 was 6.2 mM (Fig. 1) and for DHOA at pH 8.0 it was 1.5 mM (Fig. 2). No inhibitory effect of high substrate concentrations (up to 30 mM) was observed.

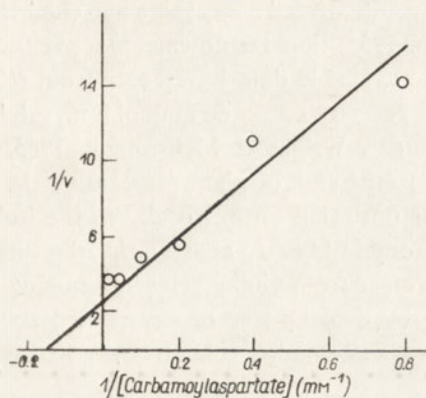

Fig. 1

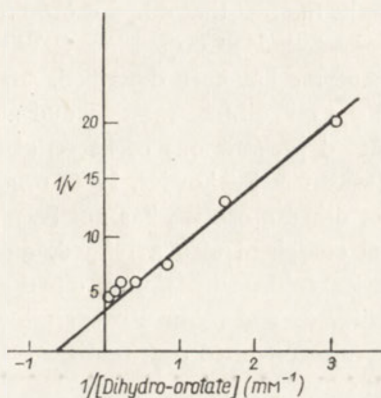

Fig. 2

Fig. 1. Lineweaver-Burk plot for dihydro-orotase-catalysed conversion of carbamoylaspartate to dihydro-orotate. *v*, μmoles of the product formed under conditions of assay system *a* (see the Materials and Methods section).

Fig. 2. Lineweaver-Burk plot for dihydro-orotase-catalysed conversion of dihydro-orotate to carbamoylaspartate. *v*, μmoles of the product formed under conditions of assay system *b* (see the Materials and Methods section).

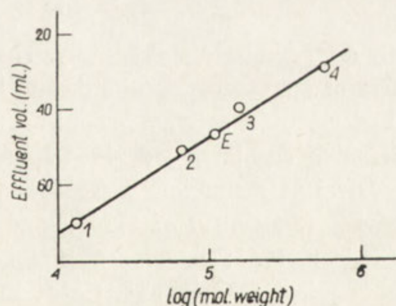

Fig. 3. Determination of approximate molecular weight of plant dihydro-orotase by gel filtration on Sephadex G-200 column. Reference proteins: *1*, ox pancreatic RNase; *2*, ox serum albumin; *3*, human γ-globulin; *4*, human fibrinogen; *E*, the studied enzyme. Experimental details are given in the Materials and Methods section.

Molecular weight. The enzyme and the reference proteins were eluted from the Sephadex G-200 column in the order illustrated in Fig. 3. From the relationship between the elution volume and the log of the molecular weight a value of about 110 000 was found for the molecular weight of plant dihydro-orotase.

Temperature and pH *optima.* The optimum temperature for the purified enzyme preparation was 60°, and the optimum pH values 6.0 for synthesis and 8.0 for degra-

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dation of DHOA; these values are practically the same as those found previously for the crude preparation (Mazuś & Buchowicz, 1966).

The possibility of non-enzymic conversion of the substrates under the incubation conditions was reasonably well excluded by suitable control experiments reported previously (Mazuś & Buchowicz, 1966) and routinely repeated in each assay.

DISCUSSION

Despite the fact that dihydro-orotase was discovered many years ago (Lieberman & Kornberg, 1954; Cooper, Wu & Wilson, 1955) thus far no extensive purification of the enzyme has been described. All the available data have come from studies carried out on cell-free extracts, and more recently on crude ammonium sulphate-precipitated preparations of bacterial (Sander, Wright & McCormick, 1965) and plant (Mazuś & Buchowicz, 1966) origin. In the case of plant preparation the presence of dihydro-orotase has not been demonstrated unequivocally as the isolated material contained also a related, dihydropyrimidinase, activity. In the present work a 320-fold purification of dihydro-orotase from young pea plant homogenate was achieved. The purified preparation proved to be free of any related activity, in particular of dihydropyrimidinase, as well as ureidosuccinase and dihydro-orotate dehydrogenase.

The data concerning the effect of pH and inhibitors, K_m values, reversibility and equilibrium of the reaction obtained for plant dihydro-orotase, are comparable, or roughly comparable with the corresponding observations reported for preparations of bacterial or animal origin. On the other hand, the stability of the plant enzyme differs markedly from any previously studied dihydro-orotase preparations. According to Beckwith, Pardee, Austrian & Jacob (1962) the bacterial enzyme is extremely unstable and, similarly, animal dihydro-orotase is rapidly destroyed by heating at 50°, as found by Bresnick & Blatchford (1964). Plant dihydro-orotase, however, survives short-time heating at 70° and may be stored at 4° for several weeks with only a moderate loss of activity. The high thermal stability and the slight inhibitory effect of mercaptoethanol indicate that disulphide bonds are not essential for plant dihydro-orotase activity, in contrast to their role for the activity of the animal enzyme (Bresnick & Blatchford, 1964).

Divalent metal ions were found to be essential for bacterial dihydro-orotase activity (Sander *et al.*, 1965), and to have no effect on the animal enzyme (Wu & Wilson, 1956; Bresnick & Blatchford, 1964). The plant enzyme apparently does not require Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} or Ni^{2+} for activity and does not respond to the presence of EDTA (Table 4). These results together with the observation that neither desalting on Sephadex G-100 column nor prolonged dialysis led to any appreciable loss of activity suggest that the enzyme does not require any low-molecular weight cofactor.

The identification of dihydro-orotase completes the search for enzymes of the orotic acid path in higher plants since aspartate transcarbamoylase (Neumann & Jones, 1962), DHOA dehydrogenase (Kapoor & Waygood, 1965), orotidine-5'-

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phosphate pyrophosphorylase (Kapoor & Waygood, 1965; Wolcott & Ross, 1967) and orotidine-5'-phosphate decarboxylase (Wolcott & Ross, 1967) have been found previously. However, the evidence for the occurrence of the whole orotate path does not exclude the possibility of other mechanisms of pyrimidine nucleotide biosynthesis being present as well.

We are grateful to Professor Dr. I. Reifer for the benefit of his advice throughout the work, and for valuable criticism on the manuscript. This work was supported by grant no. FG-Po-170 from the United States Department of Agriculture, Agricultural Research Service.

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OCZYSZCZENIE I WŁASNOŚCI DWUHYDROOROTAZY Z KIEŁKÓW GROCHU

Streszczenie

1. Dwuhydroorotaza została oczyszczona 320-krotnie z homogenatu kiełków grochu. Wyizolowany preparat był wolny od pokrewnych aktywności enzymatycznych.

2. Oczyszczony enzym katalizował odwracalną stechiometryczną przemianę *N*-karbamoilo-L-asparaginianu do L-4,5-dwuhydroorotanu. Stała równowagi tej reakcji w pH 6,0 wynosiła około 0,8. Minimalne wartości K_m wynosiły $6{,}2 \times 10^{-3}$ M dla karbamoiloasparaginianu i $1{,}5 \times 10^{-3}$ M dla dwuhydroorotanu.

3. Enzym był względnie stabilny, nie wymagał rozpuszczalnych kofaktorów i wykazywał specyficzność substratową.

4. Masa cząsteczkowa enzymu, oznaczona drogą filtracji na żelu Sephadex G-200, wynosi około 110 000.

Received 20 March, 1968.

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ACTA BIOCHIMICA POLONICA

Vol. 15 1968 No. 4

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DIHYDROPYRIMIDINASE OF PEA PLANTS

PURIFICATION AND PROPERTIES

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1. Dihydropyrimidinase from homogenates of young pea plants was purified 100-fold, and was free of dihydro-orotase activity. 2. The purified enzyme catalysed the conversion of dihydrouracil (DHU) and dihydrothymine (DHT) to carbamoyl-β-alanine (CBA) and carbamoyl-β-aminoisobutyrate (CBAIB), respectively. Attempts to separate the DHU- and DHT-degradation activities were unsuccessful. 3. The conversion of DHU to CBA proved to be reversible, although the equilibrium was strongly in favour of the DHU degradation. No cyclization of CBAIB was observed. 4. For the enzyme activity, no cofactor requirement could be demonstrated. 5. The enzyme was remarkably stable at pH 9 - 10, and much less so at pH 6 - 7. By heat treatment the enzyme could be purified 250-fold. 6. The molecular weight estimated by Sephadex G-100 gel filtration, was about 280 000.

Dihydropyrimidinase (4,5-dihydropyrimidine amidohydrolase, EC 3.5.2.2) catalyses the reversible conversion of DHU[1] and DHT to CBA and CBAIB, respectively. Thus, the enzyme plays a similar role in an alternative path of pyrimidine nucleotide biosynthesis as does dihydro-orotase (EC 3.5.2.3) in the orotic acid way. Most of the early data on dihydropyrimidinase, previously termed DHU- or DHT-hydrase, came from studies on the reductive degradation of uracil and thymine (for reviews see Reichard, 1959; Schulman, 1961). Wallach & Grisolia (1957) purified 200-fold the dihydropyrimidinase from calf liver, which catalysed the reversible degradation of both DHU and DHT. On the other hand, Campbell (1958) obtained and 80-fold purified an enzyme preparation from *Clostridium uracilicum*, which was active only toward DHU. To our knowledge, no attempt has been made to purify the enzyme from plant material. Recently the enzyme has been tentatively identified in a crude dihydro-orotase preparation isolated from pea seedlings (Mazuś & Buchowicz, 1966, 1968).

[1] Abbreviations: CA, *N*-carbamoyl-L-aspartate; CBA, *N*-carbamoyl-β-alanine; CBAIB, *N*-carbamoyl-β-aminoisobutyrate; DHOA, L-4,5-dihydro-orotate; DHT, 4,5-dihydrothymine; DHU, 4,5-dihydrouracil.

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The purpose of this paper is to describe the purification and some properties of dihydropyrimidinase from pea plants, with particular reference to its substrate specificity.

MATERIALS AND METHODS

Chemicals. 4,5-Dihydrouridine was purchased from Calbiochem. (Los Angeles, Calif., U.S.A.) and 4-methyldihydrouracil was a gift from Dr. Celina Janion. Other reagents and plant material were as specified in the accompanying paper (Mazuś & Buchowicz, 1968).

Enzyme assay. The standard incubation mixture contained 3 μmoles of the substrate and 10 μg. of protein in a total volume of 0.2 ml. of 0.1 M-diethanolamine-acetate buffer, pH 10.0. Unless otherwise stated, incubation was carried out at 37° for 1 hr. The reaction mixture was deproteinized by addition of 5 ml. of 0.3 N-$HClO_4$, followed by centrifugation. Analytical methods were the same as described previously (Mazuś & Buchowicz, 1966, 1968). Appropriate blanks were carried out to check non-enzymic degradation of the dihydropyrimidines under the incubation conditions. Corrections were necessary only for samples incubated at temperatures a bove 37°.

RESULTS

Enzyme purification

Attempts to purify plant dihydropyrimidinase according to the procedure of Wallach & Grisolia (1957) and Campbell (1958) were unsuccessful. Therefore the following procedure has been elaborated.

Crude preparation. The extract from young pea plants was prepared as described previously (Mazuś & Buchowicz, 1966). From 1500 g. of fresh material, 2240 ml. of the supernatant was obtained. To the supernatant, solid ammonium sulphate was added at 0 - 4° to 0.5 saturation, and after 30 min. the precipitate was collected by centrifugation and dissolved in 560 ml. of water.

Acetone fraction. To the crude enzyme preparation 1/2 volume of cold (-20°) acetone was added and the precipitate removed by centrifugation at 15 000 *g* for 1 min. at -10°, then to the supernatant 1/3 volume of cold acetone was added and centrifuged as before. The precipitate was dried in a stream of cold air and dissolved in 200 ml. of 0.1 M-potassium phosphate buffer, pH 7.2.

Second ammonium sulphate fraction. The acetone fraction was fractionated with ammonium sulphate and the protein precipitating between 0.3 and 0.5 saturation collected by centrifugation and dissolved in 5 ml. of 0.01 M-potassium phosphate buffer, pH 7.2.

Gel filtration on Sephadex G-100. The gel was equilibrated with 0.01 M-phosphate buffer, pH 7.2, at room temperature, poured into a column, and allowed to pack under gravity. The column (27 × 2.5 cm.) was then equilibrated with the same buffer at 4°. The second ammonium sulphate fraction was applied to the column

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and protein eluted with the same buffer, at a flow rate of 0.3 ml./min., 3-ml. fractions being collected. The enzyme was eluted between the 24th and 42nd milliliter of the effluent.

DEAE-cellulose column chromatography. The Sephadex column eluate was applied to a column (26 × 2.5 cm.) of DEAE-cellulose as described for dihydro-orotase purification (Mazuś & Buchowicz, 1968). The column was washed with 400 ml. of 0.05 M-potassium phosphate buffer, pH 7.2, and then the protein eluted with 0.1 M-buffer. The enzyme was recovered in the first 350 ml. of the effluent.

Table 1

Purification of dihydropyrimidinase from pea plants

Dihydropyrimidinase activity was assayed using dihydrouracil as substrate (see Methods), and dihydro-orotase activity with dihydro-orotate at pH 8.0 and 37° (see Mazuś & Buchowicz, 1968). The activities are expressed as μmoles of the substrate degraded/mg. of protein/hr.

Purification step	Volume (ml.)	Protein (mg.)	Dihydropyrimidinase			Dihydro-orotase
			Specific activity	Purification factor	Yield (%)	Specific activity
Extract	2 240	33 370	0.87	1	100	0.85
Crude preparation	560	14 560	1.48	2	74	0.79
Acetone fraction	200	3 840	4.06	5	54	1.17
Second $(NH_4)_2SO_4$ precipitate	5	1 125	8.80	10	34	2.26
Sephadex G-100	18	380	11.5	13	15	0.00
DEAE-cellulose	350	38	85.2	98	11	0.00

The purification procedure is summarized in Table 1. The specific activity of dihydropyrimidinase preparation was about 100 times that of the extract, and the overall yield was about 11%. The activity of the initial extracts varied from 0.6 to 1.6 μmoles of DHU degraded/mg. of protein/hr. The degree of purification obtained on the first ammonium sulphate fractionation varied over a range from 1.5 to 3.0. The efficiency of further purification steps did not vary considerably from one preparation to another. The ratio of dihydro-orotase to dihydropyrimidinase activities dropped steadily during the purification procedure, giving the final product completely free of the concomitant activity. The most efficient separation of dihydropyrimidinase from dihydro-orotase occurred on Sephadex G-100 gel filtration. If this step was replaced by dialysis, both activities remained; they could be finally separated by DEAE-cellulose column chromatography (compare the conditions for the isolation of dihydro-orotase described in the preceding paper; Mazuś & Buchowicz, 1968).

To concentrate the purified preparation, to the active eluate from the DEAE-cellulose column solid ammonium sulphate was added to 0.6 saturation, and the

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precipitate was dissolved in a minimum volume of 0.1 M-diethanolamine-acetate buffer, pH 10.0. This treatment led to no apparent changes in total or specific activities of the preparation. The concentrated enzyme solution was stored at 0 - 2° and used as a source of purified dihydropyrimidinase in the present investigation.

Properties of the enzyme

Substrate specificity. The purified enzyme was active towards DHU and DHT and, unexpectedly, also towards 4-methyldihydrouracil, a compound not found in biological material (Table 2); dihydrouridine, similarly as dihydro-orotate, was

Table 2

Substrate specificity of purified plant dihydropyrimidinase

The standard assay procedure (see the Materials and Methods section) was followed. The results are expressed as μmoles of the substrate metabolized/mg. of protein/hr.

Substrate	Enzyme activity
Dihydrouracil	85
4-Methyldihydro-uracil	32
Dihydrothymine	17
Dihydrouridine	0
Dihydro-orotate	0

Table 3

The activities toward dihydrouracil (DHU) and dihydrothymine (DHT) of ammonium sulphate fractions of the pea plant extract

The precipitated fractions were dissolved in a minimum volume of 0.1 M-diethanolamine-acetate buffer, pH 10.0, and the activity determined using about 100 μg. of protein per sample. The results are expressed as μmoles of the substrate degraded/mg. of protein/hr.

Fraction	Degradation of the substrates		
	DHU	DHT	Ratio DHU:DHT
Extract	1.5	0.8	1.9
Ppt. at $(NH_4)_2SO_4$ sat.:			
0 - 0.15	5.8	2.6	2.0
0.15 - 0.20	6.4	3.0	2.1
0.20 - 0.25	5.8	3.1	1.9
0.25 - 0.30	4.8	2.0	2.4
0.30 - 0.35	2.1	1.3	1.6
0.35 - 0.50	5.0	2.8	1.8
0.50 - 0.65	0.0	0.0	—
0.65 - 0.90	0.0	0.0	—

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not degraded. The two natural dihydropyrimidines were metabolized with different velocities. Attempts were therefore made to separate DHU- and DHT-degradation activities by gradual saturation of the initial extract with ammonium sulphate. It was found (Table 3) that all protein fractions which precipitated below 0.5 saturation catalysed the degradation of both DHU and DHT, whereas the precipitates obtained at higher saturation were inactive toward either substrate. The ratio of DHU- to DHT-degradation activity in the ammonium sulphate fractions varied over a narrow range from 1.6 to 2.4. The same ratio for the purified enzyme preparations was, however, less constant and, in general, much higher (3.5 - 5.5).

Stability. It has been demonstrated (Mazuś & Buchowicz, 1967, 1968) that dihydropyrimidinase is rapidly inactivated by heating at 70° in pH 6.0. To investigate more fully the stability of plant dihydropyrimidinase, the crude and purified preparations were dissolved in different buffers and kept either at low temperature (2°) for a long period or at 70° for a short time (Table 4). The crude preparation was found to be rapidly inactivated on storage in aqueous solution at 2°. On the

Table 4

Stability of the crude and purified plant dihydropyrimidinase

The crude and purified preparations were dissolved in water or appropriate 0.1 M-buffer to 10 mg./ml. and 0.1 mg./ml. concentration, respectively, and treated as indicated. Dihydrouracil (DHU) and dihydrothymine (DHT) degradation activities were measured as described in the Materials and Methods section, except that for the crude enzyme assay 100 μg. of protein were used. The results are expressed as a percentage of the specific activities of the untreated preparations, respectively, tested with DHU as the substrate. The actual specific activities of the untreated crude and purified enzyme preparations were essentially the same as given in Table 1.

Enzyme preparation	Solvent (water or 0.1 M-buffer)	pH	Treatment		Degradation of	
			temp.	time	DHU	DHT
Crude	Water	7.0	none		100	59
	Water	7.0	2°	5 days	0	0
	Diethanolamine-acetate	10.0	2°	5 days	90	53
	Diethanolamine-acetate	10.0	2°	20 days	65	38
	Diethanolamine-acetate	10.0	2°	30 days	63	33
	Diethanolamine-acetate	10.0	2°	60 days	51	30
	Diethanolamine-acetate	6.0	70°	5 min.	69	30
	Diethanolamine-acetate	7.5	70°	5 min.	72	36
	Diethanolamine-acetate	8.4	70°	5 min.	92	46
	Diethanolamine-acetate	9.3	70°	5 min.	95	47
	Diethanolamine-acetate	10.0	70°	5 min.	37	21
	K-phosphate-citrate	6.0	70°	5 min.	0	0
	K-phosphate	10.0	70°	5 min.	37	18
Purified	Water	7.0	none		100	28
	K-phosphate-citrate	6.0	70°	5 min.	55	16
	K-phosphate	6.0	70°	5 min.	85	18
	K-phosphate	10.0	70°	5 min.	250	95

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other hand, the same preparation was quite stable at this temperature if dissolved in 0.1 M-diethanolamine-acetate buffer, pH 10.0. Under these conditions the activity decreased gradually, with a half-life of about one month. The thermal stability of the crude preparation was dependent on the buffer used. In diethanolamine-acetate buffer, pH 9.3, the enzyme withstood 5 min. heating at 70°. At higher and lower pH values it was much less stable. At pH 10.0 it was as stable in potassium phosphate as in diethanolamine-acetate buffer. Surprisingly, however, at pH 6.0 it was relatively stable in diethanolamine-acetate but was completely inactivated in potassium phosphate-citrate buffer during the heat treatment. The purified enzyme, unexpectedly, was found to be much more stable than the crude preparation. Even in phosphate-citrate buffer, pH 6.0, it retained more than half of the initial activity after 5 min. heating at 70°, and in 0.1 M-potassium phosphate buffer, pH 10.0, the specific activity increased after heating more than twofold. On storage at 2° in phosphate or diethanolamine-acetate buffers, pH 10.0, the enzyme activity remained practically unaltered for one month.

The comparison of changes in DHU- and DHT-degradation activities revealed an apparent parallelism. The treatments that led to changes in one of them affected similarly the second (Table 4). Thus, no preferential loss of DHU- or DHT-degradation activity could be observed in either case.

Reaction products. The degradation of DHU and DHT under standard incubation conditions resulted in the formation of carbamoyl compounds. These were tentatively identified by paper chromatography as CBA and CBAIB, respectively. Quantities of the products formed corresponded to those of the substrates consumed (Table 5). Apparently no other products apart from the expected carbamoyl-amino acids were formed. In particular, we were unable to detect any formation of uracil or thymine, even in the presence of NAD.

Reversibility of the reaction. Incubation of CBA or CBAIB under standard conditions did not lead to any measurable changes in their quantity or any formation of a dihydro-compound (Table 5). However, the formation of a dihydropyrimidine could be demonstrated by incubating the enzyme with CBA under changed conditions. When the standard diethanolamine-acetate buffer was replaced by 0.1 M-potassium phosphate of pH 6.0 and the substrate concentration raised to 10 μmoles per incubation mixture (0.2 ml.) a measurable formation of a dihydro-compound accompanied by the corresponding decrease in the amount of CBA was observed. The dihydro-compound formed had the spectrophotometric properties and alkali-lability (Janion & Shugar, 1960) typical of DHU. The amount of DHU formed after 1 hr. (0.3 μmole) could not be increased by extension of incubation time or by addition of a new portion of the enzyme. Apparently, the equilibrium was approached when 9.7 μmoles of CBA and 0.3 μmole of DHU were present in the sample. Thus, the apparent equilibrium constant for conversion of CBA to DHU at pH 6.0 was approximately 0.03. At pH 10.0 no DHU formation could be demonstrated. The reverse direction of DHT to CBAIB conversion could not be unequivocally demonstrated even at pH 6.0; probably the equilibrium of this reaction is completely shifted towards DHT degradation.

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Table 5

Stoicheiometry and reversibility of the dihydropyrimidinase-catalysed reactions

The substrates indicated were incubated with the purified enzyme (25 µg. of protein) in 0.1 M-diethanolamine-acetate buffer, pH 10.0, as described in Methods, and for pH 6.0 in 0.1 M-potassium phosphate buffer.

The solvent systems for paper chromatography were as follows: *a*, propan-1-ol - conc. HCl - water (30:10:1); *b*, butan-1-ol - acetic acid - water (2:1:1) (Fink *et al.*, 1956); *c*, butan-1-ol - ethanol - formic acid - water (5:3:2:1) (Leone & Scale, 1950).

Substrate (µmoles)	pH	Found after incubation (µmoles)				R_F values for the product formed in the solvent system		
		DHU	DHT	CBA	CBAIB	*a*	*b*	*c*
Dihydrouracil, 1	10.0	0.53	—	0.45	—	0.36	0.43	0.65
Dihydrouracil, 3	10.0	1.12	—	1.86	—	0.36	0.43	0.65
Dihydrothymine, 1	10.0	—	0.84	—	0.18	0.45	0.47	0.79
Dihydrothymine, 3	10.0	—	2.28	—	0.62	0.45	0.47	0.79
Carbamoyl-β-alanine, 3	10.0	0.00	—	3.00	—			
Carbamoyl-β-alanine, 10	6.0	0.27	—	9.70	—			

Table 6

Effect of various additions to the incubation mixture on the rate of dihydrouracil (DHU) degradation

DHU was incubated with the purified enzyme and the compounds indicated under the standard conditions (see the Methods and Materials section). Enzyme tested without the additions catalysed degradation of 85.3 µmoles of DHU/mg. of protein/hr. The results are expressed as percentage of this value.

Addition	Concn. (mM)	Activity (%)	Addition	Concn. (mM)	Activity (%)
None	—	100	$HgCl_2$	1	0
$MgSO_4$	0.1	116	$AgNO_3$	0.1	3
$MgSO_4$	1	117	$AgNO_3$	1	0
$MnCl_2$	0.1	104	EDTA	1	96
$MnCl_2$	1	124	*p*-Chloromercuribenzoate	0.1	102
$CoCl_2$	0.1	116	Reduced glutathione	1	99
$CoCl_2$	1	122	2-Mercaptoethanol	1	99
$NiCl_2$	0.1	118	Cysteine	1	96
$NiCl_2$	1	130	Thioglycollate	1	96
$ZnSO_4$	1	105	Dihydro-orotate	5	102
$CuSO_4$	0.1	105	Dihydrouridine	5	90
$CuSO_4$	1	112	Citrate	10	90
$HgCl_2$	0.1	3			

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Inhibitors and activators. The response of the enzyme activity to various metal ions, thiol group-containing compounds and some other substances was studied (Table 6). Neither of the cations tested had a marked stimulatory effect: the highest stimulation, observed with 1 mM-Ni^{2+}, did not exceed 30%. Similarly, EDTA had no effect. Prolonged dialysis against 0.1 M-diethanolamine-acetate buffer, pH 10.0, caused no loss of activity, which confirms that no essential cofactors were involved. Hg^{2+} and Ag^{1+} ions in 0.1 mM concentration were strongly inhibitory. The third heavy metal ion tested, Cu^{2+}, caused no inhibition even in tenfold higher concentration. Reduced glutathione, 2-mercaptoethanol, cysteine and thioglycollate did not influence the enzyme activity. Similarly, substances structurally related to the dihydropyrimidinase substrates, dihydrouridine and DHOA, had no marked effect on the rate of DHU degradation. Citrate, which affects heat-stability of the enzyme (see above), had only a slightly inhibitory effect on its activity.

Effect of pH. The optimum pH was 10.0 for the enzyme activity assayed with DHU as substrate, and 9.5 with DHT (Fig. 1). The diethanolamine-acetate buffers allowed slightly greater rates than did any other buffers tested (diethanolamine-HCl, tris-HCl, phosphate, glycine-NaOH); the difference was never higher than 15%.

Temperature and time of incubation. The effect of temperature on the rate of DHU and DHT metabolism is shown in Fig. 2. Although 70° was the optimum temperature for both substrates, the profiles were slightly different. Both dihydropyrimi-

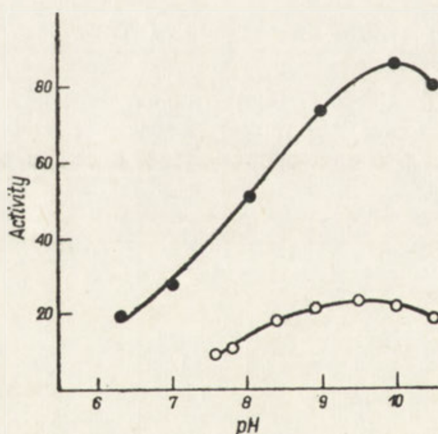

Fig. 1

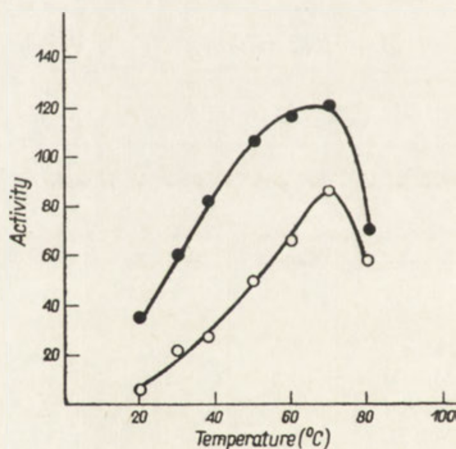

Fig. 2

Fig. 1. Effect of pH on dihydropyrimidinase activity tested with (●), dihydrouracil, and (○), dihydrothymine as substrates. The buffers used were 0.1 M-potassium phosphate for the pH values below 8.0 and 0.1 M-diethanolamine-acetate for pH $\geqslant$ 8.0. Enzyme activity is expressed as μmoles of the substrate degraded/mg. of protein/hr.

Fig. 2. Effect of the incubation temperature on dihydropyrimidinase activity tested with (●), dihydrouracil, and (○), dihydrothymine as substrates. Enzyme activity is expressed as μmoles of the substrate degraded/mg. of protein/hr.

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dinase-catalysed reactions were at 70° similarly time-dependent (Fig. 3) and the reaction rates were constant for the first 15 min.

Effect of enzyme and substrate concentration. The reaction rates for both substrates were linear functions of the enzyme concentration up to 80 μg. of protein per sample.

The K_m values calculated from Lineweaver-Burk plots (Fig. 4) were 5.6 mM for DHU and 5.9 mM for DHT.

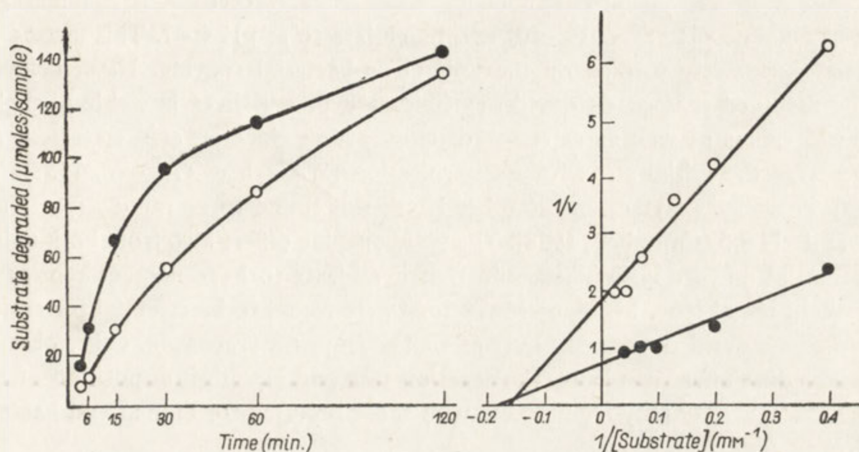

Fig. 3 Fig. 4

Fig. 3. Time-course of dihydropyrimidinase-catalysed degradation of (●), dihydrouracil, and (○), dihydrothymine. Standard reaction mixtures were incubated at 70° for the time indicated.

Fig. 4. Lineweaver-Burk plots of dihydropyrimidinase activity with varying concentrations of (●), dihydrouracil, and (○), dihydrothymine, under standard assay conditions. v, μmoles of the substrate degraded/sample/hr.

Molecular weight. This was determined after Whitaker (1963) as described in the accompanying paper (Mazuś & Buchowicz, 1968). The enzyme was eluted from the Sephadex G-200 column between the 33th and 37th ml. of the effluent, after fibrinogen but ahead of γ-globulin and dihydro-orotase. By plotting the elution volume of the marker compounds against their known molecular weight a value of about 280 000 was estimated for plant dihydropyrimidinase.

DISCUSSION

By the presented isolation procedure, dihydropyrimidinase from plant homogenates, free from dihydro-orotase activity, was purified 100-fold. The obtained preparation could be further purified 2.5-fold by heat treatment.

The purified enzyme was active towards two naturally occurring dihydropyrimidines, DHU and DHT. In this respect plant dihydropyrimidinase is similar to the animal enzyme (Wallach & Grisolia, 1957), but differs from bacterial "DHU hydrase" (Campbell, 1958) which is specific for DHU only. Despite the fact that the ratio of DHU- to DHT-degradation activities was higher for the purified plant

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enzyme preparation than for the crude one, attempts to separate these activities from each other were evidently unsuccessfull (Tables 3 and 4). Probably, plant dihydropyrimidinase does not recognise the methyl group of the substrate as it catalyses besides DHU and DHT also degradation of 4-methyldihydrouracil, while the substitution of the carboxyl group in the same ring position (DHOA) excludes interaction with the enzyme.

The stability of plant dihydropyrimidinase is quite unusual. The enzyme is much more stable in the purified than in the crude state. Moreover, it is remarkably stable at alkaline pH values (9 - 10) and much less so at pH 6 - 7. This feature has not been extensively studied on the animal or bacterial enzyme. Nevertheless, it may be assumed that crude dihydropyrimidinase of calf liver is stable at slightly acidic pH values as well as at elevated temperatures since an acid treatment and heating were the initial steps for its purification (Wallach & Grisolia, 1957). The purified bacterial enzyme, on the other hand, was found to be rapidly inactivated at 45° and pH 8.5 (Campbell, 1958). The plant enzyme differs also from both animal and bacterial dihydropyrimidinases in that it appears to have no requirement for the divalent metal ions. Mg^{2+} or Mn^{2+} ions were found to be essential for activity of the enzyme purified from calf liver as well as from *Cl. uracilicum*. Other observations, including effect of pH on the reaction rate and equilibrium position as well as K_m values, are roughly comparable with those reported for animal and bacterial dihydropyrimidinases.

Despite the unfavourable equilibrium for DHU synthesis in the isolated system the physiological role of dihydropyrimidinase may not be limited to the catabolic process only. *In vivo*, it may be also involved in an alternative path of pyrimidine ring synthesis. This seems to be quite probable as rapid conversion of CBA to uracil has been observed in intact plant tissues (Buchowicz, Reifer & Gerić, 1963).

We are grateful to Professor Dr. I. Reifer for his guidance and inspiration while pursuing this study, and for his valuable criticism during the preparation of this manuscript. This work was supported by grant no. FG-Po-170 from the United States Department of Agriculture, Agricultural Research Service.

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DWUHYDROPIRYMIDYNAZA KIEŁKÓW GROCHU

OCZYSZCZENIE I WŁASNOŚCI

Streszczenie

1. Dwuhydropirymidynazę z homogenatu kiełków grochu oczyszczono około 100-krotnie. Uzyskany preparat był wolny od aktywności dwuhydroorotazowej.

2. Oczyszczony enzym katalizował przemianę 4,5-dwuhydrouracylu (DHU) do *N*-karbamoilo-β-alaniny (CBA) oraz 4,5-dwuhydrotyminy (DHT) do *N*-karbamoilo-β-aminoizomaślanu (CBAIB). Próby rozdzielenia aktywności odpowiedzialnych za degradację DHU i DHT nie powiodły się.

3. Stwierdzono odwracalność przemiany DHU do CBA, chociaż równowaga tej reakcji była silnie przesunięta w kierunku degradacji DHU. Nie udało się wykazać cyklizacji CBAIB.

4. Aktywność enzymu nie zależała od obecności niskocząsteczkowych kofaktorów.

5. Enzym był wysoce stabilny w pH 9 - 10, a znacznie mniej w pH 6 - 7. Przez ogrzewanie można było uzyskać 250-krotne oczyszczenie enzymu.

6. Masa cząsteczkowa enzymu, oznaczona metodą filtracji na żelu Sephadex G-200, wynosiła około 280 000.

Received 20 March, 1968.

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A C T A B I O C H I M I C A P O L O N I C A

Vol. 15 1968 No. 4

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INTERACTION OF COPPER(II) IONS WITH POLYCYTIDYLIC ACID AND ITS METHYLATED DERIVATIVES

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1. The formation of Cu(II) complexes with polycytidylic acid (poly-C) was studied at various ionic strength and pH values. Non-protonated cytidylic residues are involved in the complex. The single-stranded helix, the configuration in which poly-C occurs at neutral pH, does not constitute any barrier to interaction of bases with Cu(II) ions; the double-stranded helix, the configuration in which poly-C occurs in weakly acidic media, constitutes a barrier to Cu(II) ions. Protonation of cytosine residues leads to degradation of the Cu(II) complex. 2. Poly-5-methylcytidylic acid possesses greater affinity to Cu(II) than poly-C, whereas poly-4,5-dimethylcytidylic acid does not interact with Cu(II) ions. 3. An analogy exists between the ability of the above polymers to interact with Cu(II) ions and their ability to form complexes with complementary poly-inosinic acid. It appears that in both cases the same sites in the cytosine ring are involved in hydrogen bonding with hypoxanthine residues and co-ordinated bonds with Cu(II).

Among the divalent transition metal ions Cu(II) was shown to exert a pronounced effect on the secondary structure of DNA (Eichhorn, 1962; Eichhorn & Clark, 1965; Hiai, 1965; Venner & Zimmer, 1966). The stability of DNA in the presence of Cu(II) becomes markedly reduced as reflected by a drastic decrease in its melting temperature (Eichhorn & Clark, 1965; Venner & Zimmer, 1964, 1965; Hiai, 1965; Ivanov & Minchenkova, 1965). The extent of Cu(II) induced destabilization of DNA is positively correlated with the G-C content of DNA indicating an effect of Cu(II) on G and/or C (Venner & Zimmer, 1966). This view was further substantiated by measurements of the absorption spectra of Cu(II) in the visible region in the presence of DNA and deoxynucleosides (Coates, Jordan & Srivastava, 1965; Zimmer & Venner, 1967) as well as from nuclear magnetic resonance (NMR) studies of constituent monomers (Eichhorn, Clark & Becker, 1966).

It was anticipated that additional evidence on the interaction between Cu(II) and polynucleotide bases could be obtained from investigations employing simple polymer models, e.g. synthetic homopolyribonucleotides. Indeed, the formation

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of Cu(II) complexes with poly-C, poly-I and poly-A was recently reported (Eichhorn & Tarien, 1967).

The present communication describes the spectral behaviour of poly-C[1], poly-5-MeC and poly-4,5-diMeC at various ionic strength, pH and temperature values in the presence of different Cu(II) ions concentrations. The results are considered with respect to the potential cytosine binding sites involved in the Cu(II) complex.

EXPERIMENTAL

The poly-C employed in these investigations was a commercial sample (Miles Co., Clifton, N.J., U.S.A.); it was further purified as mentioned previously (Szer & Shugar, 1966). Poly-5-MeC and poly-4,5-diMeC were prepared as described earlier (Szer & Shugar, 1966; Rabczenko & Szer, 1967); their physical characteristics were reported (Rabczenko & Szer, 1967). Absorption spectra and melting profiles were run in a Unicam SP 500 spectrophotometer equipped with a thermostated cuvette compartment (cf. Szer, 1966). No corrections were taken for thermal expansion of the liquid. For determinations in the visible region a Uvispec Hilger instrument was used. A Radiometer 22 and/or M4 pH-meter was employed.

Polynucleotide concentrations were estimated spectrophotometrically from a pancreatic ribonuclease digest. Analytical grade reagents were used throughout. Unbuffered solutions were used to prevent interaction with buffer ions, but pH was kept in the range 5.8 - 6.0, unless otherwise indicated in the text. $NaClO_4$ was used as electrolyte in all experiments since it does not complex with Cu(II) and it possesses little absorption below, and no absorption above 250 mμ. Polymer-Cu(II) complexes were prepared by mixing equal volumes of separately prepared solutions of the components at a concentration twice that of the final concentration required.

Experiments were carried out at a polymer concentration of 2×10^{-5} M to 6×10^{-5} M unless otherwise indicated in the text. Degradation of poly-C-Cu(II) complexes was tested according to Butzow & Eichhorn (1965).

Absorption spectra of poly-C and its homologues in the presence of Cu(II) *at* pH *6*

It was reported recently that Cu(II) interacts with poly-C at 0.005 M-Na^+ as indicated by extinction changes at 248 mμ (Eichhorn & Tarien, 1967). To obtain more information about the reaction sites and changes in secondary structure induced by Cu(II) the absorption spectrum of poly-C and its methylated derivatives was followed. Secondary structure properties of poly-5-MeC and poly-4,5-diMeC were described previously (Szer & Shugar, 1966; Rabczenko & Szer, 1967).

---

[1] Abbreviations: poly-C, poly-cytidylic acid; poly-5-MeC, poly-5-methylcytidylic acid; poly-4,5-diMeC, poly-4,5-dimethyl-cytidylic acid; poly-I, poly-inosinic acid; poly-A, poly-adenylic acid; other abbreviations are used according to Tentative Rules of IUPAC-IUB, 1965, *J. Biol. Chem.* **241**, 527.

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The absorption spectra presented in Fig. 1 clearly demonstrate that there is a marked increase in extinction from 220 mμ to 300 mμ upon addition of Cu(II) to saline solutions of poly-C and poly-5-MeC (in 0.04 M-$NaClO_4$, pH ~ 6). While the behaviour of both polymers follows a similar pattern it will be noted that the Cu(II) induced hyperchromicity is greater for poly-5-MeC than for poly-C at the

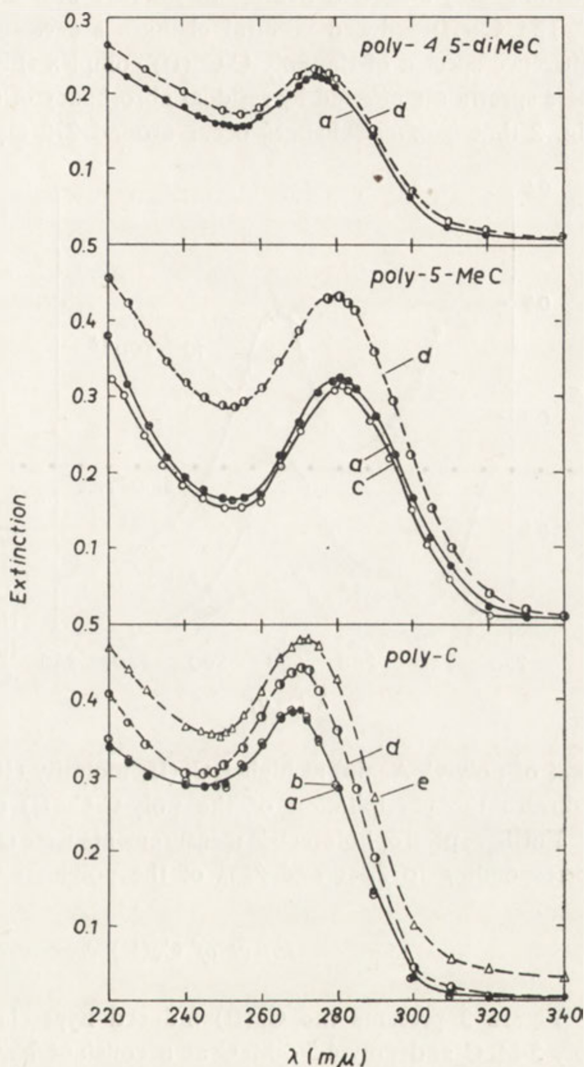

Fig. 1. Absorption spectra of poly-C, poly-5-MeC and poly-4,5-diMeC in the presence and absence of Cu(II). Conditions: 0.04 M-$NaClO_4$, pH 5.8 - 6; poly-C, 1.2×10^{-4} M; poly-5-MeC, 3×10^{-5} M; *a*, control without Cu^{2+}; *b*, 5×10^{-4} M-Mg^{2+}; *c*, 10^{-3} M-Mg^{2+}; *d*, 5×10^{-4} M-Cu^{2+}; *e*, 10^{-3} M-Cu^{2+}.

same Cu(II) concentration. Addition of Mg^{2+} produces essentially no changes in the spectra of poly-C and poly-5-MeC. The drastic change in UV spectra induced by Cu(II) suggests an interaction with the base moieties and possible changes in secondary structure of the polymers (see later Sections). At higher Cu(II) molarity (Fig. 1, curve *e*) measurements at 340 mμ reveal the appearance of turbidity testifying to the precipitation of the Cu(II)-polymer complex.

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In sharp contrast to the former two polymers, the presence of Cu(II) is virtually without effect on the spectral behaviour of poly-4,5-diMeC (Fig. 1, curves *a* and *d*). At λ_{max} the increase in extinction amounts to 2.5% and it is negligible when compared with spectral changes in poly-C and poly-5-MeC. It appears that the introduction of a methylamino group reduces almost completely the ability of the resulting poly-5-MeC derivative to interact with Cu(II).

The Cu(II) induced spectral changes are easily demonstrated by following difference spectra of the poly-C-Cu(II) complex (Fig. 2). Below 250 mμ, however, these spectra are affected by residual absorbancy of $Cu(ClO_4)_2$. It will be seen from Fig. 2 that maximal changes occur around 279 mμ, i.e. somewhat to the red of

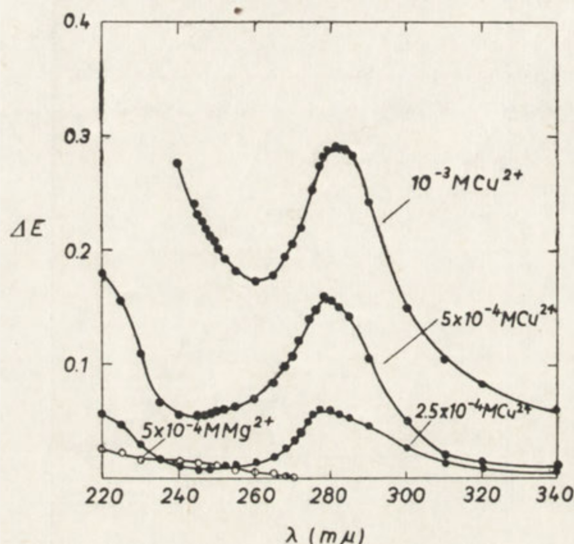

Fig. 2. Difference spectra of poly-C at various Cu^{2+} concentrations [(poly-C+Cu^{2+}) *versus* poly-C] in 0.04 M-$NaClO_4$; poly-C, concentration 1.2×10^{-4} M. Solutions containing poly-C+Cu(II) were read against corresponding poly-C solutions.

λ_{max} of poly-C. Again, at higher Cu(II) molarity (10^{-3} M), appearance of turbidity indicates the precipitation of the poly-C-Cu(II) complex.

Further spectrophotometric measurements were carried out chiefly at wavelengths corresponding to λ_{max} and λ_{min} of the polymers tested.

Effect of Cu(II) *concentration*

Figure 3 presents the Cu(II) induced hyperchromicities at λ_{max} for poly-C, poly-5-MeC and poly-4,5-diMeC at a constant $NaClO_4$ concentration. Extinction values obtained upon thermal denaturation (dotted lines) and after RNase digestion (dashed lines) are included for comparison. It is apparent that both poly-C and poly-5-MeC exhibit absorbancy increases within a distinct range of Cu(II) to polymer phosphorus ratios. For poly-C the increase in absorption starts at a ratio of about 7 Cu(II)/2 polymer P and a constant level of hyperchromicity is reached at 20 Cu(II)/2 polymer P; the Cu(II) induced effect is twice as great as thermal hyperchromicity and the final extinction value is indeed very close to that of constituent

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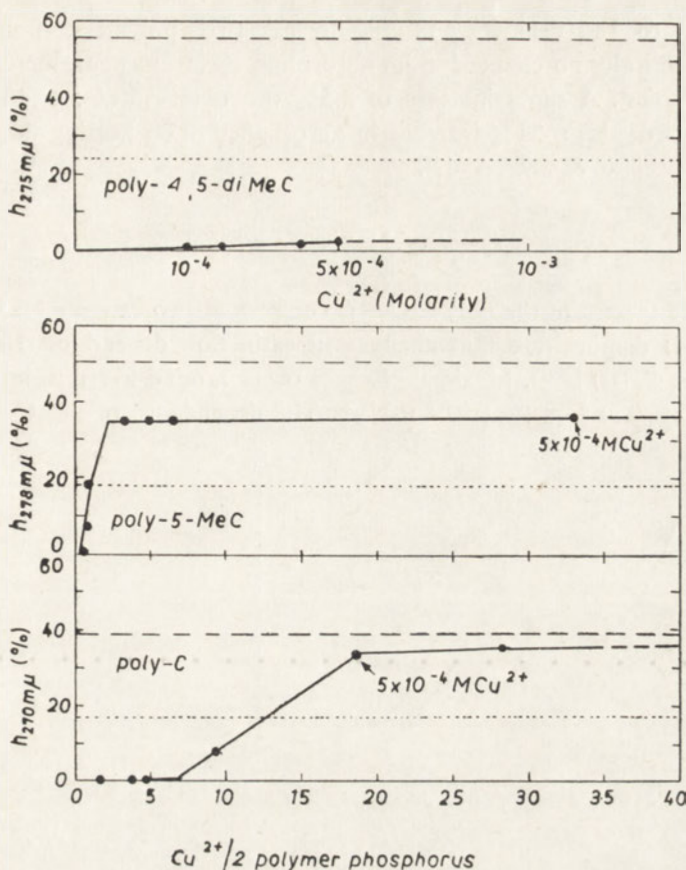

Fig. 3. Variation of the Cu(II) induced hyperchromicity of poly-C, poly-5-MeC and poly-4,5-diMeC with Cu(II) concentration. Dashed lines represent the hyperchromicity after RNase digestion and dotted lines hyperchromicity at ~95° without Cu(II).

mononucleotides. Corresponding hyperchromicity values at 248 mμ (λ_{min}) and at 279 mμ (λ_{max} of difference spectrum, cf. Fig. 2) follow a similar pattern and were not included in Fig. 3. While the behaviour of poly-5-MeC toward increasing concentration of Cu(II) is in general similar, some notable differences were observed. The increase in extinction starts here at an almost equivalent ratio of Cu(II)/polymer P and a constant level of hyperchromicity is obtained as soon as a slight excess of Cu(II) ions appears in the solution. Nonetheless, the final absorbancy value does not approach the level of constituent mononucleotides extinction as closely as in the case of poly-C. Since in neutral solutions poly-5-MeC forms a single-stranded helix with stacked bases like poly-C (Szer & Shugar, 1966), but the degree of stacking is presumably higher due to the 5-methyl group, it may be suggested that base-base interactions are affected in the presence of Cu(II) to a lesser extent than in the case of poly-C.

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Contrary to the behaviour of the former two polymers, poly-4,5-diMeC exhibited practically no changes in its absorption spectrum upon increasing Cu(II) ion concentration. A slight increase of 2.5% was observed at 5×10^{-4} M-Cu(II), to be compared with a 24% increase in absorbancy upon heating the polymer to 95° (cf. Rabczenko & Szer, 1967).

Effect of Na^+ *concentration*

Difference spectra of the poly-C-Cu(II) complex at two extreme Na^+ concentrations (Fig. 4) demonstrate that changes in extinction depend on Na^+ molarity at a constant Cu(II) level. Maximal changes occur around 279 mμ, in accord with results displayed in Fig. 2 at 0.04 M-Na^+. The dependence of the Cu(II) induced

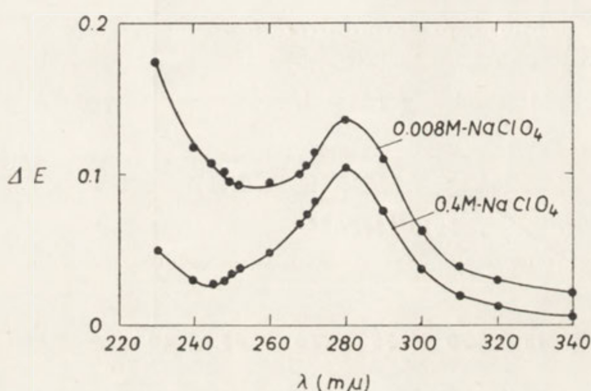

Fig. 4. Difference spectra of poly-C at 5×10^{-4} M-Cu^{2+} at two various ionic strengths; ratio of 20 Cu^{2+}/polymer P.

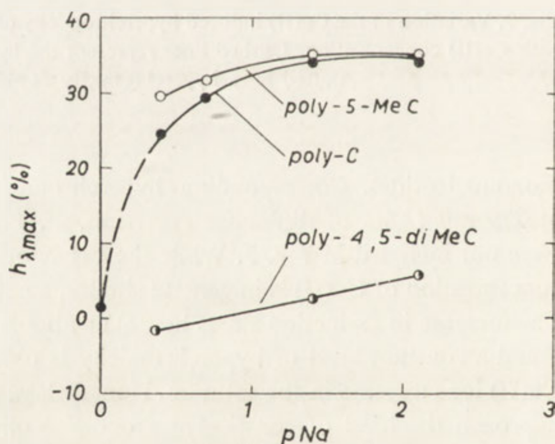

Fig. 5. Variation of Cu(II) induced hyperchromic effect of poly-C, poly-5-MeC and poly-4,5-diMeC with the concentration of sodium ions (pNa = —log MNa^+) at pH ~5.8 at 5×10^{-4} M-Cu^{2+}; at pNa = 0, NaCl was added to the poly-C-Cu^{2+} complex.

effect on Na^+ concentration for all three polymers studied is shown in Fig. 5. It will be seen that increasing Na^+ molarity decreases to some extent the Cu(II) effect. At 1 M-Na^+ (pNa 0) the Cu(II) induced effect is reduced to zero. The Cu(II) binding of poly-5-MeC appears to be much less sensitive to Na^+ concentration than the

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Table 1

The copper(II) induced hyperchromicity of poly-5-MeC at 0.4 M-$NaClO_4$

Molarity of $CuSO_4$ (M)	Ratio of Cu^{2+}/2 polymer phosphorus	Hyperchromicity* $h_{278\ m\mu}$ (%)
5×10^{-5}	3.4	—4
10^{-4}	6.6	0
2×10^{-4}	13.4	7
5×10^{-4}	34	30
10^{-3}	66	36

* Poly-C shows at 5×10^{-4} M-Cu(II) (10-15 Cu^{2+}/2 polymer P a hyperchromicity h_{270} of ~ 25%.

Table 2

Effect of electrolytes and EDTA on the binding of copper(II) ions to poly-C

Ionic conditions	Relative extinction (270 mμ)
(1) 0.04 M-$NaClO_4$	1.000
+1 M-NaCl	0.971
(2) 0.04 M-$NaClO_4$	1.000
+10 Cu^{2+}/2 polymer P	1.270
+1 M-NaCl	1.016
(3) 0.04 M-$NaClO_4$	1.000
+10 Cu^{2+}/2 polymer P	1.270
+0.01 M-$MgSO_4$	1.177 (1.033*)
(4) 0.04 M-$NaClO_4$	1.000
+10 Cu^{2+}/2 polymer P	1.270
+0.01 M-EDTA	1.091**

* Reading after 5 hr.; a precipitate appeared on further standing.

** No change with time was observed.

binding of poly-C. It will be seen from Table 1 that Cu(II) binding to poly-5-MeC occurs readily at a relatively high Na^+ concentration of 0.4 M; the change in absorption starts here at a ratio of 10 Cu(II)/2 polymer P, whereas with poly-C the Na^+ molarity must be reduced ten times in order to observe Cu(II) induced changes at a comparative ratio of Cu(II)/polymer P (cf. Fig. 3).

Very small changes in hyperchromicity are observed with poly-4,5-diMeC upon decreasing Na^+ molarity (an increase from 2.5% to 6%), in accord with results presented above.

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It is known that the denaturation of DNA by copper(II) ions can be reversed by the addition of chelating agents or by high electrolyte concentration (Eichhorn & Clark, 1965; Hiai, 1965). It will be seen from Table 2 that the Cu(II) induced effect on poly-C can also be reversed by NaCl. A similar reversibility of the Cu(II) denatured poly-(I+C) complex has been reported by Eichhorn & Tarien (1967). Partial reversibility of the Cu(II) effect on poly-C is also obtained by the addition of $MgSO_4$ or EDTA (Table 2).

Absorption-melting profiles at pH ~ *6*

Since certain metal ions are known to degrade polynucleotides (Eichhorn & Butzow, 1965; Butzow & Eichhorn, 1965), preferably at higher temperatures, it was necessary to estimate the extent of phosphodiester bond cleavage prior to melting

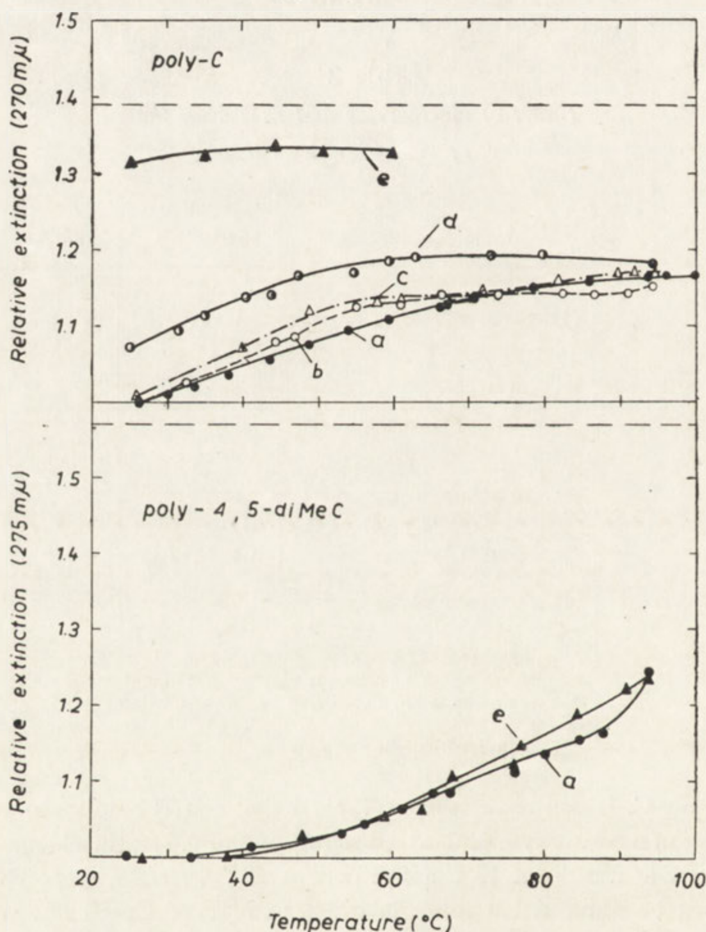

Fig. 6. Thermal denaturation profiles of poly-C and poly-4,5-diMeC in the presence of copper(II) at 0.04 M-$NaClO_4$: *a*, control without Cu^{2+}; *b*, control with 5×10^{-4} M-Mg^{2+}; *c*, 1.25×10^{-4} M-Cu^{2+}; *d*, 2.5×10^{-4} M-Cu^{2+}; *e*, 5×10^{-4} M-Cu^{2+}; dashed lines represent extinction after RNase digestion.

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experiments. Degradation of the Cu(II)-poly-C complex was tested according to Butzow & Eichhorn (1965). It was found that considerable degradation occurs upon prolonged heating; at 93° (5×10^{-5} M-Cu(II), 0.04 M-Na^+, pH ~ 6) the extent of degradation amounts to 38% and 70% after one and two hours of incubation, respectively. Since the melting profiles of the poly-C-Cu(II) complex reach a plateau at 60° (Fig. 6), it follows that they should not be significantly affected by degradation processes occurring during the experiment, at least within the temperature range from 25° to 60°. This was further confirmed by the reversibility of the poly-C-Cu(II) melting profiles at low Cu(II) concentration (cf. Fig. 7).

Heating of poly-C in saline solutions at neutral or near neutral pH produces a continuous increase in extinction, typical for single-stranded helices with stacked bases. Poly-5-MeC and poly-4,5-diMeC were shown to behave similarly (Szer & Shugar, 1966; Rabczenko & Szer, 1967). Figure 6 demonstrates that the poly-C melting curve remains almost unaffected by the addition of Mg^{2+} up to 5×10^{-4} M or Cu(II) up to 1.25×10^{-4} M (i.e. at a ratio of 4.4 Cu(II)/2 polymer P). A 16% hyperchromicity is obtained on heating to 95° and the two curves are fully reversible. Increasing the Cu(II) concentration to 2.5×10^{-4} M (8.6 Cu(II)/2 polymer P)

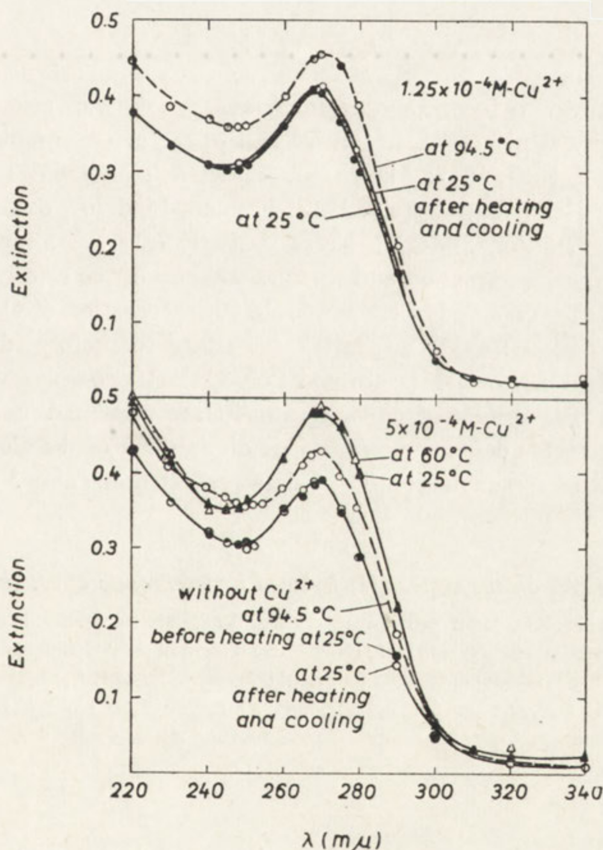

Fig. 7. Absorption spectra of poly-C after heating in the presence of Cu^{2+} (0.04 M-$NaClO_4$, pH 5.8).

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shifts the initial extinction to a higher value, and at 5×10^{-4} M-Cu(II) no further increase in extinction occurs upon heating to 60°. Above this temperature precipitation occurs. It is conceivable that at low Cu(II) molarity (4.4 Cu(II)/2 polymer P) Cu(II) is primarily involved in the interaction with polymer phosphates. At a relatively large excess of Cu(II) the complex between cytosine residues and Cu(II) is formed at room temperature and the temperature sensitive hyperchromic effect, which is due to base-base interactions, disappears.

Poly-4,5-diMeC is again insensitive to the presence of Cu(II). Even on heating, when temperature sensitive hyperchromicity disappears and the bases are presumed to be more accessible for interaction, there is no indication of complex formation. Since both poly-C and poly-4,5-diMeC possess analogous secondary structures in neutral solutions it is concluded that modification of the base moiety prevents the latter from interaction with Cu(II).

Figure 7 confirms the reversibility of the poly-C spectrum upon heating and subsequent cooling in the presence of a slight excess of Cu(II), while at higher Cu(II) concentration the spectrum becomes irreversible; it is evident that Cu(II) binding to cytosine residues prevents renaturation upon cooling.

Effect of pH

Poly-C is known to form an ordered helical structure in acid medium with maximum stability at pH ~ 4.5, i.e. where half of the cytosine moieties are protonated (Hartman & Rich, 1965; Akinrimisi, Sander & Ts'o, 1963; Ts'o, Helmkamp & Sander, 1962; Helmkamp & Ts'o, 1962; Fasman, Lindblow & Grossman, 1964; Guschlbauer, 1967). Poly-5-MeC behaves similarly (Szer & Shugar, 1966). The effect of Cu(II) on the formation and thermal stability of the acid poly-C structure was investigated. Two methods were employed for preparation of Cu(II)-containing acidic poly-C solutions: either as Cu(II) was added to preformed acid solutions of poly-C (method A), or the preformed poly-C-Cu(II) complex was acidified to the desired pH. The results of either treatment are presented in Table 3. Both series of measurements demonstrate a drastic diminishing of the Cu(II) effect upon acidification. A closer examination of the data presented in Table 3 reveals that the

Table 3

Influence of pH *on the copper(II) induced hyperchromic effect* (*h*) *of poly-C*

Conditions: 0.04 M-$NaClO_4$, room temperature. A and B indicate two methods of complex formation: A, acidic copper(II) solution was added to the acidic form of poly-C at the pH values indicated; B, the poly-C-Cu^{2+} complex was titrated to the pH values indicated; the hyperchromicities were calculated relative to the corresponding acidic poly-C solution without Cu^{2+} at the pH given in the table; Cu^{2+} concentration: A, 5×10^{-4} M; B, 4×10^{-4} M.

A	pH	6.0	5.2	4.85	4.5
	$h_{270m\mu}$ (%)	34	29	24	6
B	pH	6.1	5.7	4.1	3.8
	$h_{270m\mu}$ (%)	31	28.5	17.7	16

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Cu(II)-poly-C binding capacity is reduced much more strongly in the A-series. An 18% hyperchromic effect is found in the B-series at pH 4.1, whereas in the case of A-measurements at an even higher pH value of 4.5 the remaining hyperchromicity was only 6%. It is possible that on acidification of poly-C in the presence of Cu(II) (B-series), the Cu(II) binding reaction competes to some extent with protonation of the cytosine moiety. On the other hand, in the preformed semiprotonated structure (A-series), cytosine is less accessible for penetration by Cu(II) ions.

Thermal denaturation of the acid form of poly-C is accompanied by the release of the stabilizing shared proton (Guschlbauer, 1967). It therefore appeared useful to examine the effect of Cu(II) on the shape and reversibility of the melting profile. Figure 8 demonstrates that a relatively small excess of Cu(II) ions exerts a pronounced effect on the melting profile shifting the helix-coil transition to a lower temperature. Upon heating of acidic solutions of poly-C a decrease in extinction occurs prior to melting. This is possibly attributed to shift in λ_{max} due to partial deprotonation (Ts'o *et al.*, 1962; Guschlbauer, 1967). Deprotonation, in turn, would be expected to favour Cu(II) binding and would therefore tend to destabilize the ordered state. It will be noted that at pH 4.02 a precipitate appears above 60° (Fig. 8, curve *d*), but not in the initial part of the melting curve.

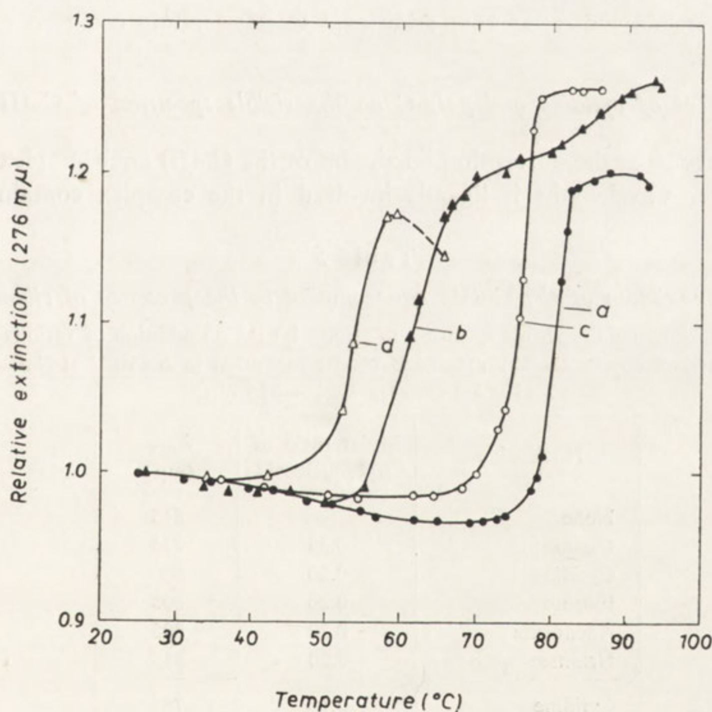

Fig. 8. Effect of Cu(II) on T_m of the acidic form of poly-C in 0.04 M-NaClO$_4$: *a*, pH 4.47 (T_m 80.5°); *b*, pH 4.47 and 10 Cu^{2+}/polymer P (T_m 63°); *c*, pH 4.02 (T_m 78°); *d*, pH 4.02 and 10 Cu^{2+}/polymer P (T_m 58°).

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Table 4

Effect of Cu(II) *on the reversibility of the acidic form of poly-C after heating*

Cu(II) concn. 1.3×10^{-4} M, approx. 10 Cu^{2+}/2 polymer P.

Ionic conditions	Temperature (°C)	Relative extinction (276 mμ)
0.04 M-$NaClO_4$, pH 4.47	25*	1.000
	92.3	1.189
	25**	0.995
0.04 M-$NaClO_4$+Cu^{2+}, pH 4.47	25*	1.000
	94.4	1.261
	25**	1.161

* Indicates measurement before heating. ** After heating and cooling.

The inference that Cu(II) binding is enhanced on heating of the acid poly-C is further supported by the data on reversibility presented in Table 4. The poly-C melting curve at pH 4.47 is completely reversible in agreement with others (cf. Guschlbauer, 1967), while in the presence of Cu(II) the change in absorbancy is partly irreversible indicating that additional Cu(II) binding occurred at elevated temperatures.

Effect of cytidine and poly-C on the visible spectrum of Cu(II)

It is known that the absorption maximum of the Cu(II) aquocomplex is shifted toward lower wavelengths if ligands involved in the complex contain nitrogen

Table 5

Absorption maximum of the Cu(II) *aquocomplex in the presence of ribonucleosides*

Absorption maximum of the visible spectrum of $[Cu(OH_2)_6]^{2+}$ on addition of various ribonucleosides at distinct copper(II) ion-nucleoside ratios; measured in 6×10^{-3} M-$NaClO_4$ plus 2×10^{-3} M-$CuSO_4$, $\lambda_{max}\sim815$ mμ.

Nucleoside	Molar ratio of Cu^{2+}/nucleoside	λ_{max} (mμ)
None	—	815
Guanosine	0.35	775
Cytidine	0.20	800
Inosine	0.20	805
Adenosine	0.20	805
Uridine	0.20	815
Cytidine	0.10	785
	0.20	800
	0.50	805
	1.0	810
	2.0	815

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(Bjerrum, Ballhausen & Norgensen, 1954). Changes in λ_{max} of the Cu(II) spectrum in the presence of major ribonucleosides are presented in Table 5. With the exception or uridine all other nucleosides tested cause a shift in λ_{max}. At a comparable Cu(II) to nucleoside ratio the order of binding capacity is G > C > I, A. This is in accord with the behaviour of deoxynucleosides [thymidine does not interact with Cu(II)] as derived from spectral changes and NMR studies (Zimmer & Venner, 1967; Eichhorn *et al.*, 1966). It is also in agreement with the known constants of complex formation between Cu(II) and ribonucleosides (Fiskin & Beer, 1965). The shift of λ_{max} for various Cu(II)/cytidine ratios is also given in Table 5. It will be seen that the shift in λ_{max} toward lower wavelengths increases on increasing cytidine concentration relative to that of Cu(II). The corresponding visible absorption spectrum exhibits a continuous hyperchromic effect as shown in Fig. 9. Poly-C exerts a similar effect (cf. curves *a* and *c*, Fig. 9), again pointing to the interaction of Cu(II) with

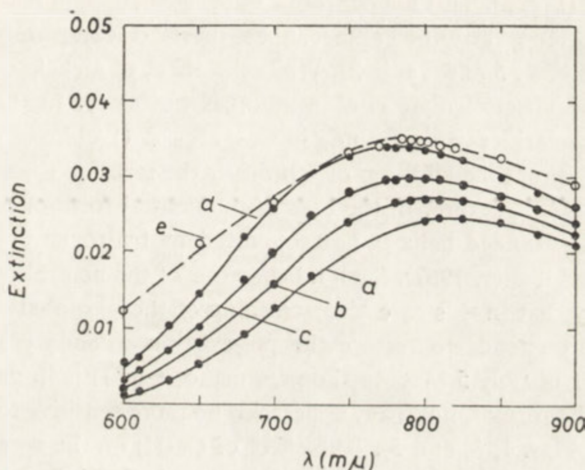

Fig. 9. Visible absorption spectra of the copper(II) aquocomplex in the presence of cytidine and poly-C. 6×10^{-3} M-NaClO$_4$ and 2×10^{-3} M-CuSO$_4$, 25°. Molar ratio of Cu^{2+}/cytidine for *a*, 0; *b*, 0.5; *c*, 0.2; *d*, 0.1. Poly-C was added to the solution of CuSO$_4$ in NaClO$_4$ to avoid precipitation and spectra were read immediately after mixing; curve *e*, 8 - 10 Cu^{2+}/2 polymer P.

cytosine moieties of poly-C. Relatively high concentrations of Cu(II) and poly-C are required for work in the visible region and the complex precipitates upon storage. The spectrum was therefore read immediately after mixing, along with a blank containing poly-C in 0.4 M-NaClO$_4$.

DISCUSSION

The behaviour of poly-C in the presence of copper(II) ions indicates the existence of a complex involving cytosine moieties. The formation of the complex depends upon the Cu(II) concentration as well as upon the ionic strength and pH of the medium. At low ionic strength (0.04 M-NaClO$_4$) and in near neutral solution

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(pH 6.0) spectral changes in poly-C are observed starting at a ratio of 7 Cu(II)/2 polymer P (cf. Figs. 1, 2, 3). The observed poly-C-Cu(II) interaction is further confirmed by the irreversibility of spectral changes occurring with poly-C upon heating in the presence of Cu(II) and subsequent cooling (Fig. 7). At a low Cu(II) concentration [1.25×10^{-4} M-Cu(II), i.e. at a ratio of 4 Cu(II)/2 polymer P, cf. Fig. 7], the thermally induced changes in the poly-C spectrum are completely reversible indicating an absence of co-ordinated bonds between Cu(II) and the base. It is possible that in this case binding occurs mainly at the phosphate site.

The interaction with Cu(II) results in an immediate increase in extinction, testifying to the destruction of the stacked poly-C structure. Indeed, if the extent of hypochromicity is taken as a relative measure of base-base interactions, it appears that cytosine moieties are completely separated in the presence of Cu(II) since the absorption of the complex approaches the value corresponding to constituent mononucleotides (Fig. 3). This hypochromic effect is partially diminished by increasing the $NaClO_4$ concentration and can be reversed completely by EDTA or 1 M-electrolyte (cf. Fig. 5 and Table 2). While the effect of a chelating agent is quite obvious, that of high electrolyte concentration is not clear. In the case of DNA where similar regularities were found, it was argued (Eichhorn & Clark, 1965; Guschlbauer, 1967) that the addition of salt drives the reaction toward the hydrogen bonded native DNA structure. In the case of the neutral form of poly-C, however, the existing single-stranded helix is not supported by hydrogen bonds (Michelson, Massoulie & Guschlbauer, 1967). Such a behaviour of the neutral poly-C form may be taken to imply that in this case also screening of the phosphate groups by high cation concentration tends to restore the pre-existing secondary structure.

The behaviour of poly-5-MeC in the presence of Cu(II) is in general similar to that of poly-C. The former, however, appears to be more sensitive to Cu(II) induced denaturation (cf. Figs. 1, 3, and 5). The effect of Cu(II) on the spectrum of poly-5-MeC is observed starting from a ratio of less than 1 Cu(II)/2 polymer P and the hyperchromic shift is essentially completed at a ratio of 2 Cu(II)/2 polymer P. No immediate explanation is forthcoming for the difference between the affinities of the two polymers for interaction with Cu(II); it will be noted, however, that an analogous difference was found for the relative affinities that the two polymers exhibit toward interaction with the complementary poly-I (Szer & Shugar, 1966). In contrast to poly-C and poly-5-MeC, the aminomethylated poly-4,5-diMeC is hardly influenced by the presence of Cu(II); again, it is noted that such a behaviour of poly-4,5-diMeC corresponds to its lack of interaction with poly-I (Szer & Shugar, 1966). This supports the view that the same sites in the cytosine moiety are involved in complementary hydrogen bonding and in co-ordinated bonds with Cu(II). A possible reason for the complex formation capacity of pyrimidines seems to be their basicity. It is known that poly-T (Szer, 1966) and thymidine (Zimmer & Venner, 1967; Eichhorn *et al.*, 1966) as well as uridine (Table 5) do not bind Cu(II) at the base residue. For the bases uracil and thymine no Cu(II)-complexes could be found whereas cytosine forms a complex with $Cu(ClO_4)_2$ (Weiss & Venner, 1965). The importance of basicity for complex formation with copper(II) was also found by

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Reinert (1966) by comparing pyrimidine, 2-aminopyrimidine, isocytosine and cytosine.

Experiments with the acidic form of poly-C also favour the view that Cu(II) interacts with the same sites where protonation occurs. The involvement of ring nitrogen is clearly implied by the competition between Cu(II) binding and protonation upon acidification of Cu(II) containing poly-C solutions (Table 3). Lowering pH to ~ 4 completely eliminates the Cu(II) effect. This is in line with the observed lack of spectral shifts in the visible spectrum of Cu(II) in the presence of protonated ribo- and deoxyribonucleosides (Zimmer & Venner, 1967). The extent of Cu(II) binding at the same pH (lower than pH 5) depends on whether Cu(II) is added to the preformed acidic poly-C structure or whether the poly-C-Cu(II) solution is acidified. Less Cu(II) is bound under the former conditions indicating that the preformed double-helical poly-C structure constitutes a barrier for Cu(II) ion penetration. On acidification of the complex, Cu(II) ions are apparently displaced from it but are capable to compete to some extent with protonation. This is somewhat similar to the behaviour of native DNA where the existence of the double-helix prevents in part binding of Cu(II) to bases. For instance, denatured DNA exhibits spectral changes upon addition of Cu(II) at room temperature, whereas native DNA shows hyperchromic changes to a lesser extent or does not interact with Cu(II) under these conditions (Eichhorn & Clark, 1965; Hiai, 1965; Venner & Zimmer, 1966; Coates *et al.*, 1965; Zimmer & Venner, to be published). At neutral pH and at low as well as at moderate ionic strength the stacked single-stranded configuration of poly-C does not seem to constitute any barrier to Cu(II) binding. Moreover, poly-5-MeC, which presumably possesses a higher degree of base stacking than poly-C itself, is less resistant to denaturation by Cu(II) than poly-C.

We are indebted to Mr. A. Rabczenko for the preparation of polyribonucleotides, and to Mrs. M. Dutkowska and Miss Ch. Radtke for excellent technical assistance.

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REAGOWANIE JONÓW MIEDZI(II) Z KWASEM POLICYTYDYLOWYM I JEGO METYLOWANYMI POCHODNYMI

Streszczenie

1. Badano powstawanie kompleksu jonów miedzi(II) z kwasem policytydylowym (poli-C) przy różnej sile jonowej i pH. W kompleksie biorą udział nieprotonowane reszty cytozynowe. Jednołańcuchowy heliks, tj. konfiguracja w jakiej poli-C występuje w obojętnym pH, nie stanowi przeszkody dla reagowania zasad z jonami Cu(II); dwułańcuchowy heliks, tj. konfiguracja w jakiej poli-C występuje w słabo kwaśnych roztworach, stanowi barierę dla jonów Cu(II). Protonacja reszt cytozynowych prowadzi do rozkładu kompleksu.

2. Kwas poli-5-metylocytydylowy posiada większe powinowactwo do jonów Cu(II) niż poli-C, natomiast kwas poli-4,5-dwumetylocytydylowy nie reaguje z Cu(II).

3. Istnieje analogia pomiędzy zdolnością wymienionych trzech polimerów do reagowania z jonami Cu(II) i do kompleksowania z komplementarnym kwasem poliinozynowym. Można przypuszczać, że te same pozycje pierścienia cytozyny biorą udział w tworzeniu wiązań wodorowych z resztą hipoksantyny i wiązań koordynacyjnych z Cu(II).

Received 21 March, 1968.

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A C T A B I O C H I M I C A P O L O N I C A

Vol. 15 1968 No. 4

LIDIA PAŚŚ and KONSTANCJA RACZYŃSKA-BOJANOWSKA

ON THE INHIBITION MECHANISMS OF VIOMYCIN SYNTHESIS BY INORGANIC PHOSPHATE

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1. The inhibiting effect of inorganic phosphate on viomycin synthesis by *Streptomyces* was associated with a decrease in total pool of free and bound amino acids and simultaneous selective accumulation of arginine, serine, aspartate — possible precursors of viomycin, and glutamate — a main component of the transamination systems. 2. Glucose-6-phosphatase, inorganic pyrophosphatase, alkaline and acid phosphatases of the *Streptomyces* strain were repressed by inorganic phosphate. 3. The kinetics of the phosphate inhibition *in vitro* of alkaline phosphatase is of a non-competitive type and that of the glucose-6-phosphatase of a mixed competitive - non-competitive type. 4. A possible role of the non-specific alkaline phosphatase in the viomycin synthesis is discussed.

Besides having a profound effect on various aspects of basic metabolism inorganic phosphates are considered to be potent regulators in the synthesis of secondary metabolites such as antibiotics (Doscocil, 1960; Nefelova, Kiseleva & Ermakova, 1967). Stimulation of fungal growth by inorganic phosphates provides a partial only explanation of a decrease in the antibiotics production and can be accounted for over a limited range of phosphate concentration (Hockenhull, 1960).

In the present work the inhibition of viomycin synthesis by inorganic phosphate has been found to be related to its effect on the activity of phosphatases and the amino acid pool. The metabolism of viomycin-producing *Streptomyces* strain was investigated under conditions of the phosphate stimulated or arrested growth and decreased or totally inhibited viomycin synthesis.

MATERIALS AND METHODS

Reagents. Lactic dehydrogenase (5 mg./ml.), glutamic dehydrogenase (20 mg./ml.), aspartic transaminase (2 mg./ml.), malic dehydrogenase (0.5 mg./ml.), peroxidase (10 mg./ml.), and glucose oxidase (1300 - 1500 units/g.), NAD^+, NADH, and glucose-6-phosphate (disodium salt) were products of C. F. Boehringer & Soehne (Mannheim, Germany). L-Serine, L-ornithine, glutamic acid, aspartic acid and

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ninhydrin were obtained fom Reanal (Budapest, Hungary); L-arginine·HCl from Sojuzchimexport (U.S.S.R.). Tris(hydroxymethyl)aminomethane was a product of Loba-Chemie (Wien-Fischamend, Austria), β-mercaptoethanol of Light & Co. Ltd. (Colnbrook, England), lysozyme (from egg white, 25 units/mg.) of Sigma (St. Louis, Mo., U.S.A.), Difco yeast extract of Difco Laboratories (Detroit, Mich., U.S.A.), Sephadex G-25 of Pharmacia (Uppsala, Sweden) and bovine blood serum albumin, cryst., of Serva (Heidelberg, Germany). Other reagents used in this work were from Fabryka Odczynników Chemicznych (Gliwice, Poland). Arginase from rat liver was prepared according to Schimke (1964) and contained 250 units/ml.

Organism. A viomycin-producing *Streptomyces* species was obtained from Dr. M. Tyc of the Division of Microbiology, Institute of Antibiotics, Warsaw.

Media and culture conditions. The medium had the following composition: water, 100 ml.; glucose, 5.0 g.; Difco yeast extract, 0.5 g.; soya flour extract, 1.5 g.; $(NH_4)_2SO_4$, 0.6 g.; $CaCO_3$, 0.5 g.; soya oil, 2.0 g.; it contained 36 μg. P_i/ml. To this medium phosphorus was added as KH_2PO_4 or DNA in different amounts. To reduce phosphate concentration, $MgCl_2$ was added to the alkalized medium; the precipitated $MgNH_4PO_4$ was centrifuged off and the medium supplemented with $(NH_4)_2SO_4$.

The *Streptomyces* strain used was grown in the 50 ml. Erlenmeyer flasks on a reciprocating shaker (240 strokes/min.) at 28° for 120 hr.

Preparation of extracts. Each 24 hr. the contents of 3 fermentation flasks were pooled, and the samples were withdrawn. The mycelium was spun down at 2000 rev./min. for 15 min. at 0° and washed twice with cold water. The mycelium was suspended in 2 volumes of 0.05 M-tris buffer, pH 7.0, containing 10 mM-β-mercaptoethanol, sonicated (M.S.E. sonicator), centrifuged, and the supernatant used for the enzyme assay. To extract the metabolites, 2 volumes of 10% (w/v) perchloric acid was added directly to the washed mycelium and homogenized in a glass homogenizer. The extract was centrifuged, neutralized with 20% KOH and separated from potassium perchlorate on a 30 min. standing in ice.

Preparation of spheroplasts. Washed mycelium was incubated for 30 min. in 1.5 vol. of 20% (w/v) saccharose containing 10 mM-β-mercaptoethanol, 30 mM-tris buffer, pH 8.0, and lysozyme (0.8 mg./g. wet wt.). Separation of the cell wall and spheroplasts was followed by the optical density measurements and microscopic examination. The spheroplasts were broken alternatively by sonication or osmotic shock.

Enzyme assay. Determination of the phosphatase activity was based on the micro estimation of the inorganic phosphate released (Chen, Toribara & Warner, 1956) from the specific enzyme substrates according to Ames, Garry & Herzenberg (1960); the composition of the incubation mixtures for determination of glucose-6-phosphatase (*a*), inorganic pyrophosphatase (*b*), alkaline and acid phosphatases (*c*) was as follows: (*a*), glucose-6-phosphate, 2 μmoles, and enzymic protein (1.25 - 1.50 μg.), in 1.0 ml. of 0.1 M-maleate buffer, pH 6.5 (Morton, 1955); (*b*), sodium pyrophosphate, 2 μmoles, enzymic protein (0.60 - 0.75 μg.) and $MgCl_2$, 1 μmole,

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in 1.0 ml. of 0.1 M-veronal buffer, pH 7.2 (Swanson, 1955); (*c*), sodium phenylphosphate, 4 μmoles, $MgCl_2$, 1 μmole, and enzymic protein (1.25 - 1.50 μg.), in 1.0 ml. of 0.1 M-bicarbonate buffer, pH 10.0, or 0.1 M-acetate buffer, pH 5.5 (Heppel, 1955). The reaction was carried out at 37° and stopped after 15 min. by the addition of 1.0 ml. of 10% trichloroacetic acid; in control, trichloroacetic acid was added before the enzyme solution. In the inactivation studies of glucose-6-phosphatase and alkaline phosphatase the liberated glucose and phenol were determined by the enzymic oxidase method (Huggett & Nixon, 1957) and the King & Armstrong's procedure (King & Wooton, 1956), respectively.

The phosphatase activity was expressed in μmoles phosphate liberated/hr./g. wet weight.

Amberlite and Sephadex separation and fractionation of amino acids. The amino acid fractions for the chemical or enzymic assays were separated from the neutralized perchlorate extract from mycelium on Amberlite IR-120 (H^+ form). Free and bound amino acids were fractionated as copper chelates on Sephadex G-25 saturated with $CuSO_4$ (Fazakerly & Best, 1965). Copper was removed on Amberlite IR-120 column (H^+ form), amino acids were eluted with 4 N-NH_4OH and the eluate evaporated *in vacuo*.

Amino acid assay. Amino acids isolated from the Amberlite column were estimated by the Rosen ninhydrin method modified by Alberti & Bartley (1963). Arginine was determined enzymically according to Greenberg (1955) with arginase prepared from rat liver (250 units/ml.), and the ornithine formed was estimated according to Chinard (1952); total amount of arginine was transformed to ornithine during a 10 min. incubation period. Determination of serine+threonine was based on oxidation of serine and threonine to glyoxylate with periodate and spectrophotometric measurement of NADH oxidized during reduction of glyoxylate to glycollate by lactic dehydrogenase (D. H. Wiliamson, 1965, personal communication). Aspartate was determined in a coupled aspartate transaminase - malate dehydrogenase spectrophotometric assay (Pfleiderer, 1963), and glutamate with glutamic dehydrogenase (Bernt & Bergmeyer, 1963).

Determination of wet and dry weight. Wet mycelium was obtained by centrifuging the culture for 15 min. at 2 000 rev./min. For determination of dry weight washed mycelium was dried to constant weight at 105°; 1 g. of wet mycelium was equivalent to 50 mg. dry weight.

Determination of viomycin. Viomycin was determined by the cylinder-plate microbiological method using *Bacillus subtilis* as a test organism (British Pharmacopea, 1963).

RESULTS

Inhibition of growth and viomycin synthesis. As can be seen from Fig. 1, the addition of 150 μg. of P_i as KH_2PO_4 per ml. of the control growth medium containing 36 μg. P_i decreased viomycin synthesis by about 90% with a simultaneous increase of mycelium growth by about 20%. The reduction of inorganic phos-

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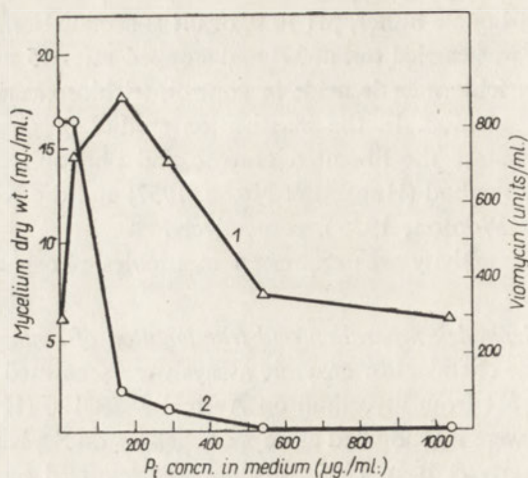

Fig. 1. Effect of inorganic phosphate on (*1*), *Streptomyces* growth, and (*2*), viomycin biosynthesis, as measured in 120 hr. culture at 37°.

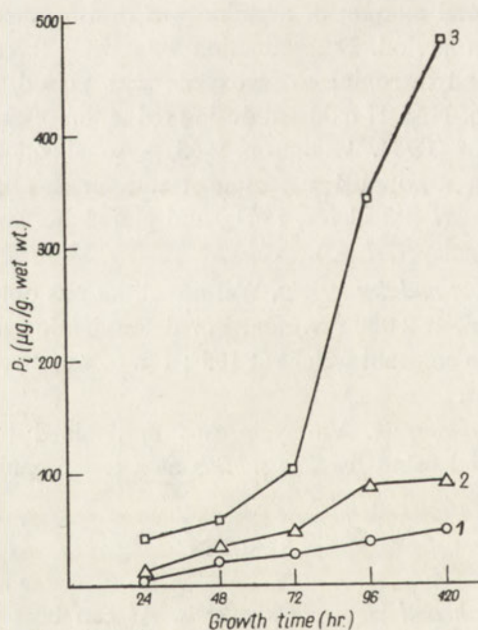

Fig. 2. Concentration of inorganic phosphate in mycelium grown on media containing (*1*), 36 µg. P_i/ml. (control); (*2*), 186 µg. P_i/ml.; (*3*), 1036 µg. P_i/ml.

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phate content in the control medium to 6 μg. P_i/ml. resulted in about twofold decrease in dry weight of mycelium without affecting viomycin synthesis. The addition of 450 μg. of P_i/ml. inhibited viomycin production totally and fungal growth by 60%. Similar results were observed when an easily hydrolysable organic phosphate (DNA) was added to the growth medium (Table 1). The addition of 150 μg. P in DNA (1.5 mg. DNA/ml.) decreased viomycin synthesis by about 60% while the addition of the same amount of P as inorganic phosphate inhibited production of antibiotic by 87%; concentration of P_i in mycelium was about 20% and in medium 4.5 times higher in cultures with KH_2PO_4 added than with DNA. A relation between concentration of inorganic phosphate and viomycin synthesis was also observed when 500 and 1000 μg. P in DNA was added to the growth medium; an about threefold higher concentration of P_i in the mycelium and medium on addition of 500 μg. P in DNA was associated with a 80% decrease in the yield of viomycin as compared with control cultures.

Basing on the results given in Fig. 1 we supplemented our control medium alternatively with 150 or 1000 μg. P_i/ml. to study the effect of inorganic phosphate on the phosphatase activities and amino acid pool under conditions of stimulated or arrested growth and decreased or totally inhibited viomycin synthesis. Changes in the concentration of inorganic phosphate in mycelium grown on the respective media are presented in Fig. 2. On control medium the concentration of inorganic phosphate in mycelium was increasing during 120 hr. from about 5 to about 50 μg./g. wet weight with regression coefficient ρ +10.8. The addition of 150 and 1000 μg. P_i/ml. resulted in an increase in regression coefficient to +21.5 and +116.1, respectively. The concentration of inorganic phosphate in mycelium after 120 hr. was twice as high (90 μg. P_i/g. wet weight) when growth was stimulated and viomycin production increased, and tenfold higher (480 μg. P_i) when growth was inhibited and mycelium was unable to produce the antibiotic.

Glucose-6-phosphatase, inorganic pyrophosphatase, non-specific alkaline and acid phosphatases. In the investigated *Streptomyces* strain, the phosphatases were localized almost exclusively in spheroplasts (Table 2). The alkaline phosphatase seems to be a firmly bound enzyme in spheroplasts, possibly membrane, since it was practically not released upon an osmotic shock, when spheroplasts were transferred from 20% saccharose to 10 mM-$MgCl_2$ solution (Nossal & Heppel, 1966). The remaining phosphate splitting enzymes were set free in 30 - 50% under the same conditions.

The activities of four investigated phosphatases in the mycelium grown on the control medium and on the media containing added inorganic phosphate are presented in Fig. 3. As can be seen (curves *1*) in control medium the maximum activity of glucose-6-phosphatase and acid and alkaline phosphatases was observed in the 48 hr. mycelium; the rise and the subsequent decline of the activity of alkaline phosphatase was especially pronounced, the activity in the 24 hr. and 96 hr. cultures being practically the same (8 μmoles P_i/hr./g. wet wt.). In contrast to this the activity of inorganic pyrophosphatase decreased rapidly during growth of mycelium

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Table 1

Effect of inorganic and organic phosphate on viomycin biosynthesis in the 120 hr. culture

Phosphorus as KH_2PO_4 or DNA was added to the control medium containing 36 μg. P_i/ml. and after 120 hr. of culture the amount of P_i was determined in the medium and in mycelium.

P added (μg./ml.)	P_i in medium (μg./ml.)	P_i in mycelium (μg./g. wet wt.)	Viomycin (units/ml.)
None	4.8	41.2	915
as DNA			
150	1.5	93.6	320
500	13.4	139.0	200
1000	23.2	167.0	120
as KH_2PO_4			
150	8.1	113.0	122
500	160.0	242.0	62

Table 2

Localization of phosphatases in the mycelium

The cell wall and spheroplasts were separated after treatment of mycelium with lysozyme. The enzymic activities were determined as described in Methods.

Enzyme	Expt. no.	Activity (μmoles P_i/hr./g. wet wt.)				
		Whole cell sonicated	Cell wall	Spheroplasts		Cell wall + spheroplasts
				sonicated	osmotic shocked	
Glucose-6-phosphatase	I	8.00	0.00	8.00		8.00
	II	10.00	0.80	9.20		10.00
	III	7.60	1.60	6.00	3.20	7.60
Inorganic pyrophosphatase	III	48.00	1.00	46.40	12.80	47.40
Alkaline phosphatase	I	27.20	2.72	25.20		27.92
	II	26.40	3.60	22.40		26.00
	III	26.40	3.12	20.80	2.40	23.92
Acid phosphatase	I	4.32	0.00	4.16		4.16
	II	6.00	0.00	6.40		6.40
	III	4.64	0.00	4.32	2.00	4.32

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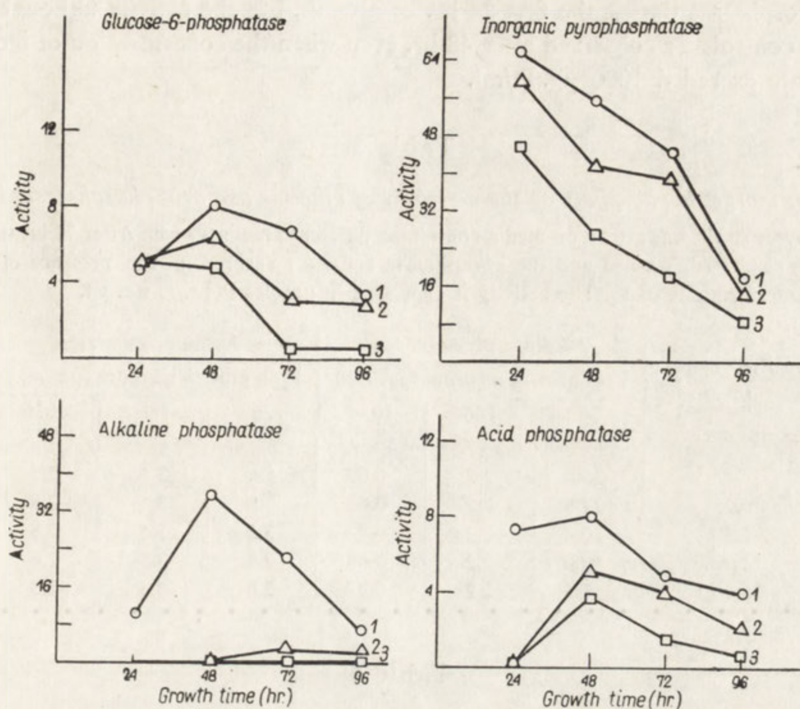

Fig. 3. Effect of inorganic phosphate present in the growth medium on the activity of phosphatases. P was added to the medium as KH_2PO_4; (*1*), 36 μg. P_i/ml. (control); (*2*), 186 μg. P_i/ml.; (*3*), 1036 μg. P_i/ml. The activity is expressed in μmoles P_i released/hr./g. wet wt.

to the value amounting to 30% of that observed in the 24 hr. cultures (64 to 20 μmoles P_i).

Inorganic phosphate added to the medium in both concentrations (150 and 1000 μg./P_i/ml., curves *2* and *3*, resp.) repressed the activity of the investigated enzymes. The activity of glucose-6-phosphatase was only slightly affected in the 24 hr. cultures, and a distinct decrease was observed after 72 hr. The character of changes in the activity of inorganic pyrophosphatase was not influenced by inorganic phosphate added to the medium, the activity in the 96 hr. culture was decreased only by about 10% when 150 μg. P_i/ml. was added; the addition of 1000 μg. P_i/ml. caused a 30% decrease after 24 hr. and about 50% decrease after 96 hr. of growth.

Under conditions of decreased viomycin synthesis (150 μg. P_i added/ml.) no activity of alkaline phosphatase could be detected in cultures after 24 and 48 hr. of growth, a low activity appeared, however, in the later hours, in the stage of viomycin production. In the media containing above 1000 μg. P_i/ml. total inhibition of the viomycin synthesis was associated with a lack of the enzymic activity throughout the whole growth period.

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Inorganic phosphate added to the medium abolished similarly the activity of acid phosphatase during the first 24 hr. of growth, but the activity although lower than in controls was observed after 48 hr. even when the concentration of inorganic phosphate exceeded 1000 μg. P_i/ml.

Table 3

Effect of inorganic phosphate on the activities of alkaline and acid phosphatases in vitro

Streptomyces strain was grown on media containing different amounts of P_i. After 72 hr. the mycelium was separated, washed and the phosphatase activity measured in the presence of various concentrations of P_i. The activity is expressed in μmoles P_i/hr./g. wet wt.

P_i concn. (mM)	Alkaline phosphatase			Acid phosphatase		
	P_i in growth medium (μg./ml.)			P_i in growth medium (μg./ml.)		
	36	186	1036	36	186	1036
—	24.6	2.2	0.03	8.4	3.5	2.5
0.25	24.6	2.2	0.03	7.9	3.5	2.8
2.50	20.4	2.2	0.03	3.5	3.5	2.8
12.00	10.0	2.2	0.03	2.9	3.5	2.6
25.00	7.5	2.2	0.03	2.6	3.5	2.5

Table 4

The kinetics of alkaline phosphatase and glucose-6-phosphatase, and phosphate inhibition

Enzyme	K_m	K_i
Alkaline phosphatase	3.7×10^{-5} M	2.3×10^{-2} M
Glucose-6-phosphatase	2.0×10^{-4} M	8.0×10^{-2} M

It was found that the concentration of the 7 min.-phosphate labile compounds, the majority of which are substrates for the non-specific phosphatases, was increased in the 72 hr. mycelium from 11.7 to 31 and 68 μmoles P_i/g. wet wt. when media were supplemented with 150 and 1000 μg. P_i/ml., respectively. The activities, however, of the acid and alkaline phosphatases were not induced on a 2 hr. incubation of the 72 hr. control culture with glycerophosphate or phenylphosphate (0.1 M) in contrast to the phosphatases of HeLa cells induced by substrates under the same conditions (Cox, Gilbert & Griffin, 1967). On the contrary, an about 50% decrease in these activities was noted when substrates were added either directly to the growth medium or to a washed mycelium suspended in 0.05 M-tris buffer, pH 7.0. Thus the reappearance of the activities of non-specific phosphatases in the later hours of mycelium growth on the phosphate-rich media was not the result of substrate induction, but it could be a consequence of an adaptation process, manifested *in vitro* in a complete resistance to inorganic phosphate of the enzymes derived from mycelium kept on the phosphate-rich media (Table 3).

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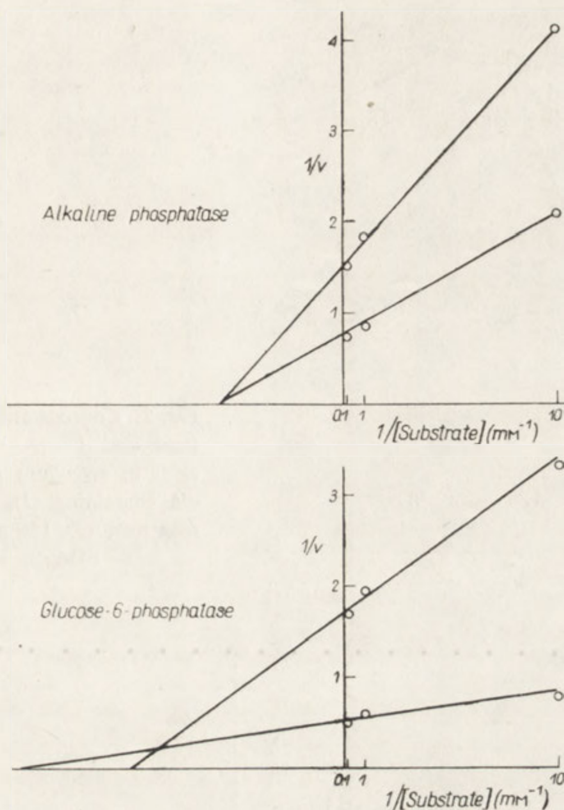

Fig. 4. The Linewear and Burk plots representing phosphate inhibition of alkaline phosphatase and glucose-6-phosphatase. Concentration of inorganic phosphate 0.1 M.

Inorganic phosphate besides its effect on formation of phosphatases showed a product inhibition type of inactivation of these enzymes *in vitro*. The corresponding K_i values for alkaline phosphatase and glucose-6-phosphatase are given in Table 4 together with K_m values for their respective substrates: phenyl phosphate and glucose-6-phosphate. The alkaline phosphatase was about 4 times more sensitive than glucose-6-phosphatase to inorganic phosphate when incubated for 15 min. at 37° in 0.05 M-tris buffer containing 0.01 - 1 M-KH_2PO_4. The kinetics of the phosphate inhibition of alkaline phosphatase was of a non-competitive type, and that of glucose-6-phosphatase of a mixed competitive - non-competitive type (Fig. 4.).

Amino acid pool. During growth of mycelium an about 60% increase in the total pool of free and bound amino acids was observed (Fig. 5). The amount, however, of arginine, serine+threonine and aspartate, possible precursors of viomycin, remained practically unaltered (Fig. 6) (ρ close to 0). The concentration of glutamate, the most active component of transamination systems, fell down from 4.5 to 3.3 μmoles/g. wet wt. ($\rho = -0.350$). Inorganic phosphate added to the medium diminished the increase in the amino acid pool both of the free and bound amino

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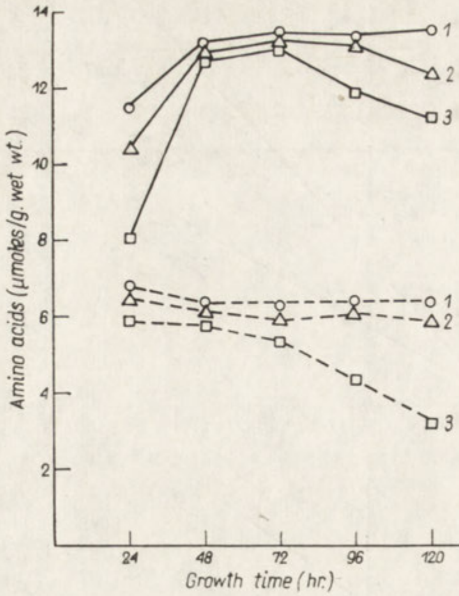

Fig. 5. Concentration of (———), total and (— — —), free amino acids in mycelium grown on media containing (*1*), 36 μg. P_i/ml. (control); (*2*), 186 μg. P_i/ml.; (*3*), 1036 μg. P_i/ml.

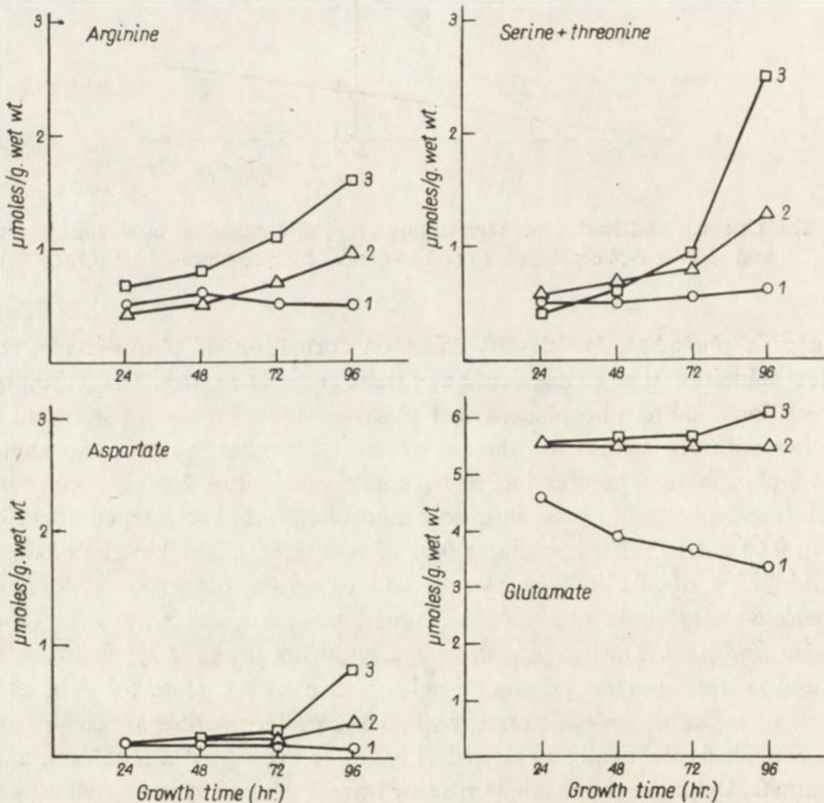

Fig. 6. Concentration of arginine, serine + threonine, glutamate and aspartate in mycelium grown on media containing (*1*), 36 μg. P_i/ml. (control); (*2*), 186 μg. P_i/ml.; (*3*), 1036 μg P_i/ml.

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acids. The regression coefficient of the amino acid concentration against fermentation time (in days) was +1.50, +1.11 and +1.05 in control medium and in media supplemented with 150 and 1000 μg. P_i/ml., respectively. In contrast to these changes in the total amino acid pool the addition of inorganic phosphate resulted in accumulation of the examined amino acids. The corresponding regression coefficients for control and the two phosphate supplemented media were: for arginine −0.035, +0.196 and +0.328; for serine+threonine +0.009, +0.178, +0.652; for aspartate 0.000, +0.017, +0.153; and for glutamate −0.350, −0.046, +0.132. The amount of glutamate in the 96 hr. mycelium grown on medium containing 1036 μg. P_i/ml. was practically the same as in the 24 hr. mycelium.

DISCUSSION

Neither the biochemical mechanisms responsible for viomycin synthesis nor even the structure of this peptide antibiotic is fully known (Bowie, Cox, Johnson & Thomas, 1964; Dyer, Kellog, Nassar & Streetman, 1965; Kitagawa, Sawada, Miura, Ozasa & Taniyama, 1968). Spaeren, Frøholm & Laland (1967), working with the cell-free extracts of *Bacillus brevis* synthesizing Gramicidin S found that the synthesis of this peptide is an entirely different process from that of protein biosynthesis. The explanation therefore of the action of factors such as inorganic phosphate inhibiting viomycin synthesis is open to discussion. This holds especially for the relation between the decrease in viomycin synthesis and repression of phosphatases by inorganic phosphate. In the viomycin-producing *Streptomyces* strain the repression involved all four investigated phosphate splitting enzymes: glucose-6-phosphatase – an enzyme regulating glucose metabolism (Weber, 1963), inorganic pyrophosphatase – promoting nucleic acid synthesis (Kornberg, 1962) and both non-specific alkaline and acid phosphatases. This kind of phosphate inhibition of a negative feed-back type was described for a number of phosphatases (Torriani, 1960; Gallant & Stapleton, 1964). The examination, however, of the phosphatase activities in the mycelium cultured under conditions of phosphate stimulated or arrested growth and decreased or totally inhibited viomycin synthesis (186 or 1036 μg. P_i/ml.) enabled some differentiation concerning the role of phosphatases in the metabolism of viomycin-producing strain of *Streptomyces*. The activity of inorganic pyrophosphatase is connected with the stage of vigorous growth of mycelium and declines steeply during the period of intensive synthesis of the antibiotic in the later hours of incubation. Under conditions of decreased or totally inhibited viomycin synthesis this activity is still high, ranging from 40 - 60 μmoles P_i/hr./g. wet weight, i.e. 90 and 70% of the activity found in the control 24 hr. mycelium (Fig. 3). The activities of the remaining phosphatases may be connected with the metabolism in the stationary phase of growth, which is the stage of viomycin production. It is interesting that under investigated conditions the activity of alkaline phosphatase shows the closest relation to the viomycin biosynthesis. The analogous enzyme in *Streptomyces griseus* is indispensable for the

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transamidination step in the synthesis of streptidine, a component of streptomycin (Walker & Walker, 1967). The occurrence of guanidine groups in the viomycin molecule (Bowie *et al.* 1964; Dyer *et al.*, 1965; Kitagawa *et al.*, 1968) may involve a similar enzymic system. More information is, however, required concerning this activity under various conditions of viomycin biosynthesis and in variants derived from a single genetic population.

An almost exclusive localization of phosphatases in the spheroplasts (Table 2) is a characteristic feature of the viomycin-producing strain as compared with *Escherichia coli* (Malamy & Horecker, 1961 and 1964) and *Streptomyces* strains producing other antibiotics (Ruczaj, Paśś, Sawnor-Korszyńska & Raczyńska-Bojanowska, 1968). In yeasts alkaline phosphatase was found inside the cell, while an acid phosphatase on its surface (Weinberg & Orton, 1964).

A selective action of inorganic phosphate on the metabolism of amino acids in the investigated *Streptomyces* strain should be emphasized. An accumulation of possible precursors of viomycin, arginine, serine+threonine, aspartate and glutamate, under conditions of general decrease of amino acid pool may suggest participation of the phosphate-sensitive enzymic systems utilizing amino acids in viomycin biosynthesis.

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MECHANIZMY HAMOWANIA SYNTEZY WIOMYCYNY PRZEZ NIEORGANICZNY FOSFORAN

Streszczenie

1. W czasie zahamowanej syntezy wiomycyny przez nieorganiczny fosforan obserwowano spadek całkowitej puli wolnych i związanych aminokwasów z równoczesnym selektywnym nagromadzeniem argininy, seryny i asparaginianu — prawdopodobnych prekursorów wiomycyny, oraz glutaminianu — głównego składnika układów transaminujących.

2. U badanego szczepu *Streptomyces* produkującego wiomycynę fosfataza glukozo-6-fosforanowa, nieorganiczna pyrofosfataza, oraz nieswoiste fosfatazy alkaliczna i kwaśna podlegają represji przez nieorganiczny fosforan.

3. Hamowanie alkalicznej fosfatazy przez nieorganiczny fosforan *in vitro* ma charakter niekompetytywny, a fosfatazy glukozo-6-fosforanowej mieszany.

4. Przedyskutowano możliwy udział fosfatazy alkalicznej w procesie biosyntezy wiomycyny.

Received 2 April, 1968.

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ACTA BIOCHIMICA POLONICA

Vol. 15 1968 No. 4

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THE EFFECT OF IODINATION ON THE ACTIVITY AND STRUCTURE OF ACID PHOSPHOMONOESTERASE FROM HUMAN PROSTATE

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1. Acid phosphomonoesterase of human prostate was irreversibly inhibited by iodine monochloride at pH 8.1. 2. Tartrate, a competitive inhibitor, protected partially the enzyme from ICl inactivation. 3. The change in the spectrum of the iodinated enzyme and filtration on Sephadex G-100 indicated that a part of tyrosine residues became monoiodinated and the protein dissociated into smaller fragments. 4. At 0.05 mM-ICl concentration, which caused inhibition of the enzyme by about 75%, 14 of the 42 tyrosine residues of the protein were monoiodinated. 5. Tyrosinase did not oxidize tyrosine residues, nor did it inactivate the enzyme.

During the last few years much attention has been paid to chemical modification of enzymes and its effect on their activity. One of the methods for modifying the structure of protein is iodination by I_3^- (Hartdegen & Rupley, 1964; Covelli & Wolff, 1966; Cowgill, 1966; Dube, Roholt & Pressman, 1966; Roholt & Pressman, 1967) or by ICl (Izzo, Bale, Izzo & Roncone, 1964; Simpson & Vallee, 1966). During iodination, depending on the kind of protein and conditions of the reaction, cysteine, methionine and tryptophan residues are oxidized, and tyrosine and histidine are halogenated (Cha & Scheraga, 1963; Filmer & Koschland, 1964).

In our previous papers we have demonstrated that acid phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) of human prostate contains two non-reactive SH groups; blocking of these groups by Ag^{1+} ion in the presence of tris as complexing agent, leads to partial only loss of activity of the enzyme (Domański, Konieczny & Ostrowski, 1964). Analysis of the amino acid composition of the purified enzyme indicates a rather high content of tyrosine and tryptophan (M. Derechin, W. Ostrowski & E.A. Barnard, in preparation), and that is why this protein exhibits a high extinction coefficient at 280 mμ (Boman 1958). The above properties of the prostatic phosphomonoesterase prompted us to study the effect of iodination on the activity and physico-chemical properties of the enzyme. Iodine monochloride at a concentration of 0.1 mM at pH 8.1 abolished

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completely within a few minutes the activity of the phosphomonoesterase; higher concentrations caused fragmentation of the enzyme molecule, which was probably due to dissociation of the protein into subunits.

MATERIAL AND METHODS

Acid phosphomonoesterase from hypertrophic human prostate was obtained according to the procedure described by Ostrowski & Tsugita (1961) and modified by Ostrowski (1968). The enzyme was homogeneous on Sephadex G-100 gel filtration, on polyacrylamide gel electrophoresis and on ultracentrifugation. The specific activity of the enzyme amounted to about 1.65 m-mole of *p*-nitrophenyl liberated from *p*-nitrophenylphosphate per 1 mg. protein, in 1 min. at 25° under standard conditions (Ostrowski, 1968).

Iodine monochloride was prepared according to Izzo *et al.* (1964). Tyrosinase from mushrooms was from Sigma Chem. Comp. (St. Louis, Mo., U.S.A.), crystalline bovine serum albumin from British Drug Houses, Ltd. (Poole, England), and Sephadex G-25 and G-100 from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade and were used without further purification.

The activity of acid phosphomonoesterase was determined according to the procedure described previously (Ostrowski & Tsugita, 1961), except when otherwise indicated in the text.

Spectrophotometric titration of native and iodinated enzyme was carried out using an Uvispec spectrophotometer (Hilger & Watts, England) and a pH-meter, model PM 22 (Radiometer, Copenhagen, Denmark) provided with a glass electrode, type G 220B. Enzyme solution, 0.12 - 0.18 mg. protein/ml. of 0.1 M-KCl, was introduced to a spectrophotometric cell of 1 cm. light path, then 1 N-KOH was added using an Agla type microburette (Burroughs, Wellcome & Co., London, England). After determination of the pH of the solution, the extinction at 290, 295, 305 and 325 mμ was measured at 20° against an appropriate control sample. Then the pH of the solution was checked and the extinction readings repeated. Molar extinction coefficients for tyrosine, monoiodotyrosine and di-iodotyrosine were taken according to Wolff & Covelli (1966) for the appropriate wavelengths.

Protein was determined as nitrogen, using Nessler reagent, after oxidation with concentrated sulphuric acid.

RESULTS

Spectral properties of acid phosphomonoesterase

The absorption spectrum of the enzyme at pH 6.45 shows a maximum at 276 mμ and a minimum at 250 mμ (Fig. 1). At pH 13 in 0.1 N-NaOH two characteristic maxima appeared at 285 and 291 mμ, due to shifting of the absorption by phenolic tyrosine group.

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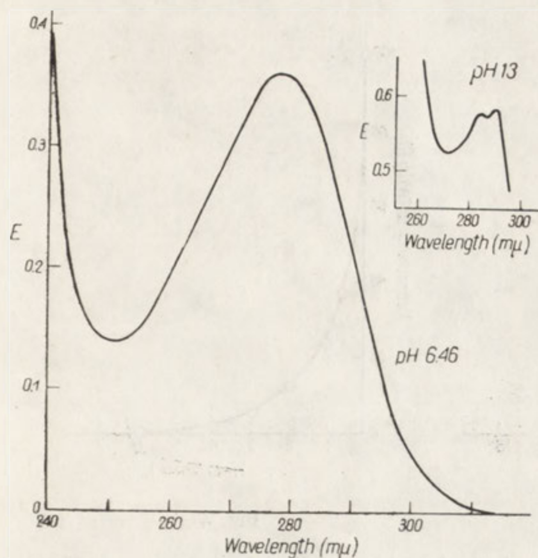

Fig. 1

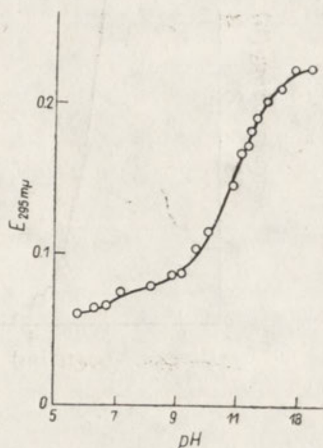

Fig. 2

Fig. 1. The ultraviolet absorption spectrum of acid phosphomonoesterase at pH 6.46 (0.145 mg. of protein/ml.) and at pH 13 (0.270 mg./ml.). The enzyme was dissolved in water and adjusted to the required pH with 1 N-NaOH.

Fig. 2. Spectrophotometric titration of phosphomonoesterase at 295 mμ. A 2.7 ml. sample containing 0.12 mg. of protein/ml. in 0.1 M-KCl was titrated with 1 N-KOH.

The results of spectrophotometric titration of phosphomonoesterase are shown in Fig. 2. The extinction at 295 mμ, up to pH 9, rose slowly whereas beginning with pH 10 the increase was sharp up to pH about 12; further changes in extinction were much slower. The midpoint of the titration curve was at p*K* 10.8. From the increase in extinction over a pH range from 9.3 to 13 and taking the change of molar extinction coefficient due to ionization of tyrosine, $\Delta\varepsilon_{295}$, equal to 2400 (Wolff & Covelli, 1966), the maximum number of tyrosine residues was calculated to be 43.5; this is in close agreement with the value of 42 tyrosine residues obtained from amino acid analysis (M. Derechin *et al.*, in preparation).

Effect of iodination on the activity of acid phosphomonoesterase

Kinetics of iodination. The enzyme was incubated in 0.05 M-tris-HCl buffer, pH 8.1, with different ICl concentrations. A 20 mM-ICl solution in 5 M-NaCl solution was adjusted to pH 5.5 with 1 N-NaOH and appropriately diluted with 2 M-NaCl, then added to the enzyme solution. The final volume of the incubation mixture was 0.1 ml. and the concentration of the enzyme 0.03 mg./ml. After 3 min. at 20° and pH 8.1, the reaction was stopped by adding 200 μl. of 30 mg% crystalline bovine serum albumin in 0.05 M-citrate buffer, pH 5.5. Then the activity of the enzyme was determined using 20 mM-*p*-nitrophenylphosphate in 50 mM-citrate buffer, pH 5.5, containing 30 mg% of albumin; the incubation time being 1 min.

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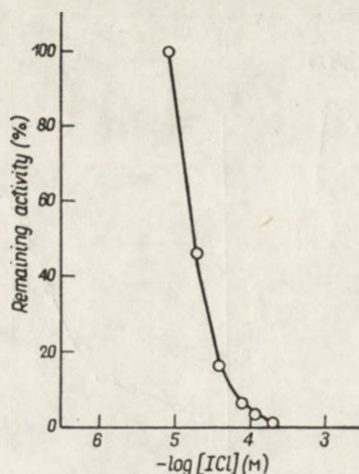

Fig. 3

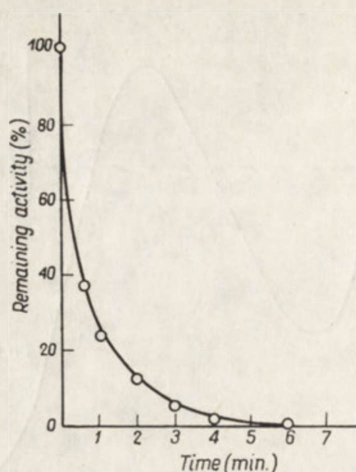

Fig. 4

Fig. 3. The effect of ICl concentration on the inhibition of phosphomonoesterase. The enzyme, 0.03 mg./ml., was incubated for 3 min. at room temperature with ICl at pH 8.1. The reaction was stopped by the addition of a 30 mg% solution of albumin in citrate buffer, pH 5.5 (200 µl./0.1 ml. of the incubation mixture). Then the activity of the enzyme was measured.

Fig. 4. Time-course of phosphomonoesterase inactivation by 0.1 mM-ICl at pH 8.1. Other conditions were as described for Fig. 3.

and temp. 37°. The decrease in enzyme activity as a function of ICl concentration is shown in Fig. 3. At 0.02 mM-ICl concentration, the activity of phosphomonoesterase was inhibited by about 50%. Complete inhibition of the enzyme occurred at 0.2 mM-ICl concentration. The course of the curve is distinctly biphasic: up to 0.05 mM-ICl, which caused an inhibition of the enzyme by about 80%, the relationship is linear and the slope of the curve is steep; the inhibition is probably due to iodination of amino acid residues at the active centre or its immediate vicinity. Higher concentrations of ICl resulted in deeper changes in the enzyme molecule, which are reflected in the further course of the curve in Fig. 3; this will be more fully discussed below.

The time-course of inactivation of the enzyme by 0.1 mM-ICl is shown in Fig. 4. The reaction was very rapid and complete inhibition of enzyme activity was obtained after about 4 min. Dialysis or filtration on Sephadex G-25 did not lead to reactivation of the iodine-inhibited enzyme, indicating the irreversibility of the inhibition.

Reversible inhibition of phosphomonoesterase by ICl *in the presence of tartrate.* L-Tartrate is a competitive inhibitor of acid phosphomonoesterase of human prostate (Tsuboi & Hudson, 1955), and K_i is 8 mM at pH 5 (Kilsheimer & Axelrod, 1957). To study the effect of competitive inhibition on the inhibition by iodine monochloride, the enzyme was incubated at room temperature in 0.05 M-tris-HCl buffer, pH 8.1, in the presence of 0.1 M-L-tartrate for 10 min., then ICl was added to a final concentration of 0.04 mM, and the mixture incubated for a further 10 min. The enzyme was also incubated under the same conditions alone or with one of the two inhibitors. The reaction was stopped by adding 200 µl. of 30 mg% albumin solution in 0.05 M-

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citrate buffer, pH 5.5, per 0.4 ml. of the sample, and enzyme activity was measured directly and after filtration at 3° on a Sephadex G-25 column (20 × 1 cm.) equilibrated with the above buffer containing albumin (Table 1). In the sample iodinated in the presence of tartrate, after removal of the excess of the inhibitors by gel filtration, about a half of the activity that in the absence of tartrate would have been inhibited by iodine, was recovered; this indicates that the enzyme was in part protected by tartrate from iodine inactivation. The results obtained in this experiment indicate that iodine monochloride at 0.04 mM concentration either modified amino acids at the active centre of the enzyme, or acted on these sites of the molecule of which native conformation is involved in binding with substrate or the competitive inhibitor.

Table 1

The effect of L-*tartrate on inactivation of acid phosphomonoesterase of human prostate by iodine monochloride*

The enzyme in 0.05 M-tris-HCl buffer, pH 8.1, was incubated 10 min. at room temperature, alone, with 0.1 M-tartrate, with 0.04 mM-ICl, or with both 0.1 M-tartrate and 0.04 mM-ICl, and the activity was determined directly and after filtration on Sephadex G-25

Inhibitor added	Enzyme activity (% of the control)	
	before filtration	after filtration
None	100	100
Tartrate	0	100
ICl	60	60
Tartrate+ICl	0	78

The effect of tyrosinase on the activity of phosphomonoesterase. It has been shown by several workers (see Cory & Frieden, 1967) that tyrosinase modifies different proteins and can also affect the activity of some enzymes, for instance aldolase and alcohol dehydrogenase. The mechanism of tyrosinase action on protein consists in oxidation of tyrosine residues to *o*-dioxyphenyl derivatives, which usually leads to changes in the spectrum of the modified protein (Dabbous, 1966). Taking into account the above demonstrated effect of iodination on the activity of prostate phosphomonoesterase, which probably consists in blocking of tyrosine residues, it seemed interesting to study the effect of tyrosinase. The reaction was carried out at pH 5.5 in 0.05 M-citrate buffer, and at pH 7.5 in 0.066 M-phosphate buffer. Phosphomonoesterase, 10 - 30 μg., was incubated in a final volume of 0.6 ml. with 100 μg. of tyrosinase at 8° or 25°, up to 48 hr. No changes in the activity were observed in any of the samples studied, irrespective of the ratio of tyrosinase to phosphatase and time of incubation. In this respect acid phosphomonoesterase of human prostate behaves similarly to ribonuclease, glutamate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, trypsin, catalase and some other enzymes which under

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similar conditions were not inactivated by tyrosinase (cf. Cory & Frieden, 1967). These results may indicate that tyrosine residues of acid phosphomonoesterase are situated in such a way that they are inaccessible to tyrosinase.

Physico-chemical properties of the iodinated enzyme. Changes in the ultraviolet absorption spectrum of phosphomonoesterase after iodination with ICl indicate the modification of tyrosine residues. In Fig. 5 are presented the spectra of the native enzyme, of the enzyme partly iodinated (inhibited by about 40%, at a Tyr: ICl ratio of 1:0.4), and of the enzyme completely inhibited (Tyr:ICl ratio 1:16). As it may be seen, the maximum absorption of the iodinated enzyme was displaced toward the longer wavelengths, and the absorption in the region of 300 - 340 mμ distinctly increased due to the formation of iodotyrosine (Houssier & Fredericq, 1967). At a large excess of ICl, the spectrum reflected the changes occurring in the secondary and tertiary structure of protein.

Spectrophotometric titration at 290 mμ (Fig. 6) of the phosphomonoesterase iodinated at pH 8.1 indicated that in the partly inactivated enzyme (possessing about 25% of the initial activity) the tyrosine residues were titratable at lower pH

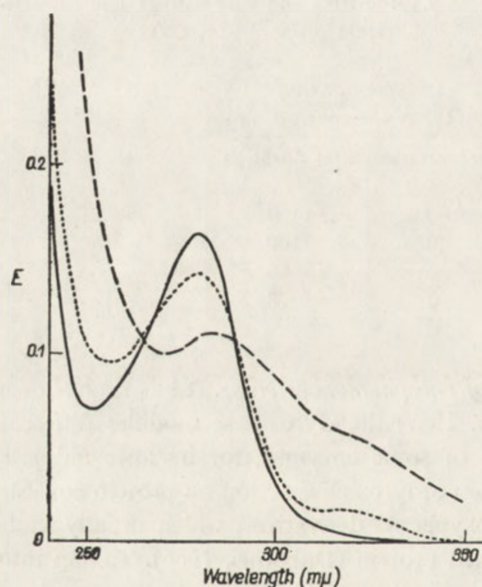

Fig. 5

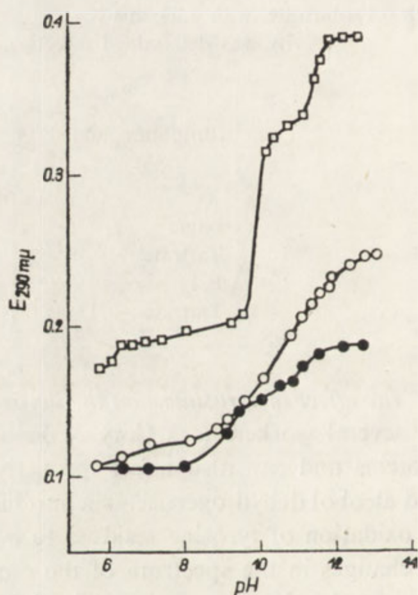

Fig. 6

Fig. 5. The absorption spectrum of (———), native phosphomonoesterase; (- - -), the enzyme iodinated with 0.02 mM-ICl (Tyr:ICl ratio, 1:0.4, inhibition 40%); and (— — —) the enzyme iodinated with 7.6 mM-ICl (Tyr:ICl ratio, 1:16, complete inhibition). The enzyme at a concentration of about 0.1 mg. of protein/ml. was iodinated in 0.05 M-tris-HCl buffer, pH 8.1, and then dialysed against 0.05 M-tris-HCl buffer, pH 6.5, containing 0.1 M-KCl.

Fig. 6. Spectrophotometric titration of phosphomonoesterase at 290 mμ. (○), Native enzyme (0.12 mg. of protein/ml.); (●), enzyme iodinated with 0.05 mM-ICl at pH 8.1 (0.12 mg. of protein/ ml.); (□), enzyme iodinated with 0.1 mM-ICl in 0.1 M-acetate buffer, pH 5 (0.18 mg. of protein/ml.). All samples before titration with 1 N-KOH were dialysed against 0.1 M-KCl solution.

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values than in the native enzyme. Titration at 305 mμ (Fig. 7) over a pH range from 7.1 to 9.3 indicated that about 14 tyrosine residues were monoiodinated (for calculation, $\Delta\varepsilon_{305}$ for monoiodotyrosine was taken to be 4000, after Wolff & Covelli, 1966). The course of the titration at 325 mμ of the iodinated enzyme indicated that there was no formation of di-iodotyrosine (pH range from 4.8 to 7.1). Titration at 305 mμ of the enzyme iodinated at pH 5 in 0.1 M-acetate buffer demonstrated that under these conditions iodination of tyrosine did not occur (Fig. 7). At the same time, the enzyme iodinated at pH 5 did not lose the enzymic activity.

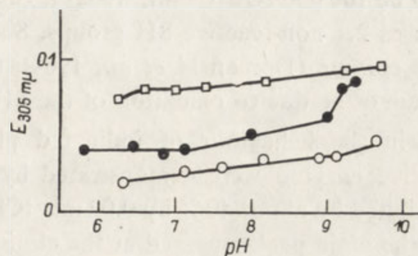

Fig. 7. Spectrophotometric titration of phosphomonoesterase at 305 mμ. (○), Native enzyme; (●), enzyme iodinated at pH 8.1; (□), enzyme iodinated at pH 5.0. Other conditions as for Fig. 6.

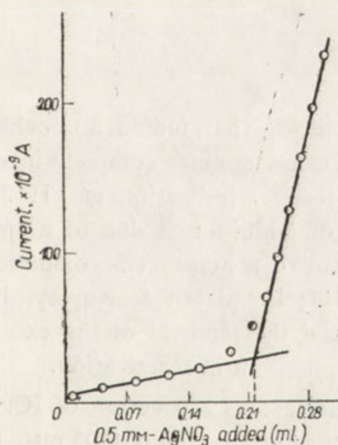

Fig. 8

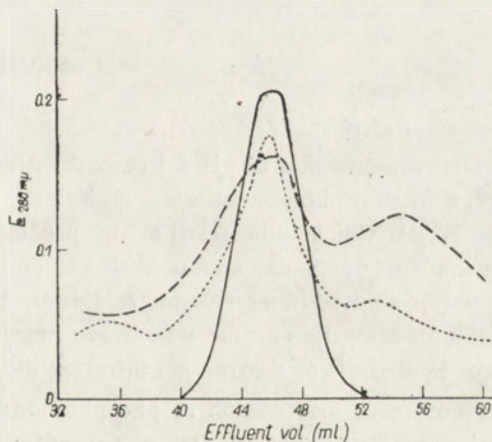

Fig. 9

Fig. 8. Amperometric titration of SH groups of the phosphomonoesterase iodinated at alkaline pH. A sample of 5 mg. of protein in 2.4 ml. of tris-HCl buffer, pH 8.1, was iodinated with 0.08 mM-ICl, dialysed against 0.17 M-tris-HNO_3 buffer, pH 7.9, containing 0.5% of sodium dodecyl sulphate, and titrated with 0.5 mM-$AgNO_3$ (Domański *et al.*, 1964).

Fig. 9. Sephadex G-100 gel filtration of phosphomonoesterase. (———), Native enzyme; (- - -), enzyme iodinated at pH 8.1 with 0.02 mM-ICl (Tyr:ICl ratio, 1:0.4); (— — —), enzyme iodinated at pH 8.1 with 7.6 mM-ICl (Tyr:ICl ratio, 1:16). The filtration was carried out on a 110×1 cm. column at 3° in 0.05 M-tris-HCl buffer, pH 6.5, containing 0.1 M-KCl. Samples applied to the column contained about 1 mg. of enzyme protein per 1 ml. of the solution. Fractions of 0.8 ml. were collected at a rate of 3.2 ml./hr.

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To determine whether oxidation of thiol groups does occur during iodination, the phosphatase iodinated at pH 8.1 for 3 min. was submitted to amperometric titration. About 5 mg. of the iodinated protein, possessing 20% of the initial activity, was dialysed against water and the solution was transferred to an electrolytic vessel; after addition of sodium dodecyl sulphate, titration with Ag^{1+} ion was carried out in 0.17 M-tris-HNO_3 buffer, using a rotating platinum electrode as described previously (Domański *et al.*, 1964). From Fig. 8 it can be seen that 0.22 ml. of 0.5 mM-$AgNO_3$ was used per enzyme sample (5 mg.); taking the molecular weight of the protein to be 100 000 (Ostrowski, 1968) it can be calculated that one enzyme molecule possesses 2.1 non-reactive SH groups. Since the same value was obtained for the native enzyme (Domański *et al.*, 1964), the decrease in enzyme activity on iodination cannot be due to oxidation of the SH groups.

Distinct differences in the behaviour of iodinated phosphomonoesterase as compared with the native enzyme were demonstrated by filtration on Sephadex G-100. The enzyme inhibited by about 40% by 0.02 mM-ICl at pH 8.1 showed two protein peaks (Fig. 9); the main peak emerged at the elution volume of the native enzyme, and the second, inactive one emerged later. The enzyme treated with an excess of iodine monochloride, and completely inhibited, gave two inactive fractions: one at the elution volume corresponding to the native enzyme, and another, emerging from the column much later.

DISCUSSION

The results of the presented experiments indicate that iodine monochloride at 0.1 mM concentration at pH 8.1 caused within a few minutes complete inactivation of acid phosphomonoesterase of human prostate. Iodination at pH 5 was without effect, which indicates that the process of inhibition is due to a specific modification of particular amino acids rather than to a non-specific oxidation of some amino acid residues (Anson & Edsall, 1947; Hartdegen & Rupley, 1964). The inhibition of the enzyme was irreversible, and the removal of the excess of inhibitor by dialysis or dextran gel filtration did not result in reactivation.

The course of inactivation of phosphomonoesterase as a function of ICl concentration indicates that at lower concentrations, up to about 0.05 mM, there occurred a specific blocking of the amino acids directly involved in the catalytic function of the enzyme. At higher concentration of ICl, about 0.2 mM, greater changes in the structure of the protein took place, as evidenced by spectral changes (Fig. 5) and by the appearance of a polypeptide of lower molecular weight on Sephadex G-100 gel filtration. It should be noted that the action of sodium dodecyl sulphate, and low or high pH value also leads to dissociation of the protein into subunits (M. Derechin *et al.*, in preparation). It seems interesting that the enzyme completely inhibited by treatment with 0.1 mM-ICl was still able to react with the specific antibody (Ostrowski, Weber & Rybarska, 1966).

Among different specific agents that modify amino acid residues, tyrosinase

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is of special importance for determination of the role of tyrosine in the biological activity of proteins. Cory & Frieden (1967) classified proteins with respect to the reactivity to tyrosinase into three groups: "(1), proteins in which the biological activity is lost, either partially or completely, on oxidation by tyrosinase; (2), proteins, in which tyrosyl groups are oxidized, but there is no loss of biological activity, and (3), proteins which are completely resistant to oxidation by tyrosinase". From the results obtained in the present work it appears that acid phosphomonoesterase of human prostate belongs to the third group of proteins, in which the biological activity is unaffected by tyrosinase. Spectrophotometric titration of phosphomonoesterase (Fig. 2) demonstrated that practically no tyrosine groups are titratable below pH 10; this suggests that they are buried in the folded protein molecule. Thus the inaccessibility of tyrosine groups to tyrosinase may be due to steric configuration of the enzyme molecule. This configuration, however, permits the iodination of tyrosine residues leading to modification of the structure of the protein molecule, with concomitant loss of enzyme activity.

Spectral changes of phosphomonoesterase iodinated at pH 8.1 distinctly indicate the formation of monoiodotyrosine, although it is known that in alkaline medium iodination of protein may also lead to formation of iodo-derivatives of histidine, as it has been observed for chymotrypsinogen (Glazer & Sanger, 1964) and ribonuclease A (Covelli & Wolff, 1966). An increase in absorption in the region of 300 - 340 mμ of the iodinated phosphomonoesterase as compared with the native enzyme (Fig. 5) indicates the modification of tyrosine residues. On the basis of spectrophotometric titration of the enzyme inhibited in 75% by ICl at pH 8.1 it has been found that 14 tyrosine residues, of the 42 probably present per molecule, are monoiodinated. On the other hand, from titration at 325 mμ over a pH range from 4.8 to 7.1 it follows that di-iodotyrosine was not formed (Edelhoch, 1962; Wolff & Covelli, 1966). Of course, simultaneous formation of iodohistidine derivatives cannot be ruled out, but specific reagents modifying histidine residues, such as iodoacetate and iodoacetamide, as well as photo-oxidation in the presence of Rose Bengal and methylene blue had no, or a very slight, effect on enzyme activity (K. Bobrzecka, J. Rybarska & W. Ostrowski, in preparation). Hence, the participation of histidine in the catalytic function of acid phosphomonoesterase of prostate seems to be but slight.

Thiol groups of protein may be oxidized on iodination (Fraenkel-Conrat, 1955). Acid phosphomonoesterase of prostate contains two unreactive thiol groups, but their blocking with a large excess of Ag^{1+} ions leads to partial only loss of enzyme activity (Domański *et al.*, 1964). To check the possible changes in SH groups during iodination, the iodinated and inhibited by 80% enzyme was titrated amperometrically with Ag^{1+} ions, after unmasking of SH groups by treatment with sodium dodecyl sulphate. Two titratable thiol groups were demonstrated, similarly as it has been found in the non-iodinated protein, showing that the loss of enzyme activity on iodination was not due to modification of SH groups.

The participation of tyrosine residues in the catalytic function of phosphomono-

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esterase is suggested also by the experiments in which tartrate was applied as the competitive inhibitor. The presence of tartrate reduced the inhibition by iodine. Thus it appears that the competitive inhibitor protected the iodine-binding site.

It is known that tyrosine residues in proteins may be phosphorylated and also react with di-isopropylphosphorofluoridate (DFP) (Murachi, Inagami & Yasui, 1965). Greenberg & Nachmansohn (1965) have demonstrated that acid phosphomonoesterase of human prostate undergoes slow inactivation in the presence of DFP, the inhibition being reversible after removal of the inhibitor by dialysis. The above authors were unable to demonstrate the presence of phosphoserine in prostate phosphatase, and it appears that its active centre differs from that of alkaline phosphatase of *E. coli* (Pigretti & Milstein, 1965) and from other sources (Barman & Gutfreund, 1966). This behaviour of the enzyme seems to support the view that tyrosine rather than serine is involved in the active centre of phosphomonoesterase.

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WPŁYW JODOWANIA NA AKTYWNOŚĆ I STRUKTURĘ KWAŚNEJ FOSFOMONOESTERAZY STERCZU LUDZKIEGO

Streszczenie

1. Kwaśna fosfomonoesteraza sterczu ludzkiego jest nieodwracalnie hamowana przez JCl przy pH 8.1.

2. Inkubowanie enzymu z winianem jako kompetytywnym inhibitorem przed dodaniem JCl, powoduje obniżenie wpływu hamującego JCl na aktywność fosfomonoesterazy.

3. Zmiana widma zjodowanego enzymu oraz filtracja na Sephadexie G-100 wskazuje, że JCl powoduje zamianę części reszt tyrozylowych do monojodotyrozyny oraz rozpad cząsteczki białka na mniejsze fragmenty.

4. Przy stężeniu JCl 5×10^{-5} M, powodującym zahamowanie enzymu w ok. 75%, 14 reszt tyrozylowych białka na 42 obecnych występuje w postaci monojodotyrozyny.

5. Działanie tyrozynazy na fosfomonoesterazę nie powoduje utlenienia reszt tyrozylowych ani utraty aktywności biologicznej enzymu.

Received 16 April, 1968.

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A C T A B I O C H I M I C A P O L O N I C A

Vol. 15 1968 No. 4

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SULPHOSALICYLIC ACID-SOLUBLE PROTEINS AND THEIR DISTRIBUTION IN SUBCELLULAR FRACTIONS OF RABBIT KIDNEY

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1. From nuclei of rabbit kidney, lysine-rich histone soluble in 0.15 M-sulphosalicylic acid (SSA) was isolated; it was found to correspond on paper electrophoresis to the fastest-moving cathodic fraction of SSA-soluble proteins of rabbit kidney, and on carboxymethylcellulose column chromatography to the proteins of peak *III*. This fraction exhibits RNA depolymerase activity and is digested by trypsin.

Studies on proteins soluble in sulphosalicylic acid (SSA), isolated from kidney (Mejbaum-Katzenellenbogen & Wieczorek, 1966), skeletal muscle (Mejbaum-Katzenellenbogen, Kozar & Maskos, 1967) and other tissues have demonstrated their basic character. Recently, Maskos & Mejbaum-Katzenellenbogen (1968), using SSA-extraction and the tannin-caffeine procedure, have isolated lysine-rich histone from calf thymus. A similar technique was applied by Rzeczycki, Grudzińska & Hillar (1962) to isolate from hog kidney mitochondria basic proteins which inhibited the activity of enzymes participating in electron transport (Rzeczycki, 1963), uncoupled oxidative phosphorylation (Hillar, 1965) and displayed depolymerase activity toward RNA (Bardoń & Rzeczycki, 1966).

The present work concerns the distribution in subcellular fractions of SSA-soluble proteins of rabbit kidney, which are compared with histone isolated from nuclei.

MATERIAL AND METHODS

Subcellular fractions. Rabbits were killed by bleeding, then the kidneys were excised, cooled to -6°, and cleaned. The isolation of subcellular fractions was carried out at 0 to 10°. The kidneys were minced, homogenized in a Waring blendor with a tenfold volume of 0.25 M-sucrose - 1.8 mM-$CaCl_2$, and the homogenate filtered through three layers of gauze to remove tissue debris. The nuclei were sedimented by centrifugation for 10 min. at 300 - 400 *g*. The nuclei still remaining in the supernatant were centrifuged off for 10 min. at 600 - 800 *g* and discarded. The mito-

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chondria were collected by centrifugation at 20 000 *g* for 10 min. The nuclear and mitochondrial fractions were washed three times with cold 0.25 M-sucrose solution.

The SSA-soluble proteins from whole kidney, nuclei, mitochondria and the post-mitochondrial supernatant were obtained and concentrated as described in a previous paper (Mejbaum-Katzenellenbogen & Wieczorek, 1966).

Histones from nuclei were isolated according to Wolfe & McIlwain (1961). The kidney was homogenized with a tenfold volume of 0.32 M-sucrose - 3 mM-$CaCl_2$ at pH 7 and 0 - 10°. The homogenate was filtered through three layers of gauze and centrifuged at 300 - 400 *g* for 10 min. at 0°. The nuclei pellet was washed four times with sucrose, and twice with 0.14 M-NaCl, pH 7; then ten volumes of 0.2 N-H_2SO_4 was added and centrifuged at 800 *g* for 30 min. at 0°. The supernatant was treated with 95% ethanol (cooled to -10°) to a final concentration of 20% (v/v), stirred and left overnight at -5°. The sediment of arginine-rich histone was collected by centrifugation at -5° and washed three times with four volumes of 95% ethanol. From the supernatant the lysine-rich histone was precipitated with ethanol, which was added to a final concentration of 50% (v/v), and the sediment washed with 95% ethanol. The two histone fractions were dissolved in small volumes of water. The scanty undissolved sediments were centrifuged off and discarded.

Digestion of SSA-soluble proteins of nuclei by trypsin and pepsin was carried out for 30 min. as described previously (Mejbaum-Katzenellenbogen, Wilusz & Polanowski, 1966). The protein was dissolved in phosphate buffer, pH 7.6, or in 0.06 N-HCl to a concentration of about 100 μg./ml. For the reaction, 0.9 ml. of protein and 0.1 ml. of enzyme solution of appropriate concentration, were taken. The incubation was carried out at 30° and stopped by adding the tannin reagent.

Activity of RNA depolymerase was determined as described by Bardoń & Rzeczycki (1966). The sample, 1 ml., contained 1.5 mg. of RNA, 10 μg. of SSA-soluble protein from nuclei, and 0.05 M-phosphate buffer, pH 6.9. After 15 min. at 37°, 3 ml. of acetic acid - *tert.*butanol (1:2, v/v) was added. In the supernatant the extinction was determined at 260 mμ.

Analytical methods. Protein was determined by the turbidimetric tannin micromethod (Mejbaum-Katzenellenbogen, 1955). Protein and polypeptide solutions were concentrated by the tannin-caffeine procedure (Mejbaum-Katzenellenbogen, 1959). Carboxymethylcellulose (CM-cellulose) column chromatography was performed according to Rzeczycki, Grudzińska, Hillar & Wszelaki-Lass (1963).

Paper electrophoresis was carried out in 0.15 M-citrate - phosphate buffer, pH 4.4, for 6 hr. at a voltage of 200 v. The protein, 200 - 400 μg., was applied at the middle of a strip (28 × 3 cm.) of Schleicher-Schüll paper no. 2043a. The electrophoretograms were stained with bromophenol blue in tannin reagent according to Mejbaum-Katzenellenbogen & Dobryszycka (1959), and the fractions were eluted with 0.1 N-NaOH.

Free-boundary electrophoresis was performed in the "Electrophoresgerät 35" (C. Zeiss, Jena, German Democratic Republic), in citrate - phosphate buffer, pH 4.4, at 3 mA, 85 v and 4°, for 2 hr.

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Reagents: Tannin, pure, containing 80% of gallotannin (imported from U.S.S.R., lot 20/66, by Cefarm, Katowice, Poland); trypsin, activity 20 000 Fuld-Gross units/g. (Merck A. G., Darmstadt, Germany); pepsin, 3 times crystallized (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); Whatman carboxymethylcellulose (fine) (W. & R. Balston Ltd, England). Yeast RNA was prepared according to Mallette & Lamann (1953). Other reagents were of analytical purity, produced in Poland.

RESULTS

SSA-soluble proteins isolated from whole rabbit kidney submitted to paper electrophoresis in citrate - phosphate buffer, pH 4.4 (Fig. 1a) gave five fractions moving toward the cathode (K_1 - K_5); the fraction possessing the lowest mobility had a tendency to move toward the anode. The percentage distribution of the SSA-soluble proteins is shown in Table 1. It should be noted that the intensity of staining of the respective fractions was not proportional to the amount of protein eluted with 0.1 N-NaOH. On the average, 26.7% of protein was found in fraction K_5, which had the greatest affinity toward bromophenol blue, whereas 35.4% was found in the rather weakly staining fraction K_2.

Table 1

Distribution of electrophoretic fractions of sulphosalicylic acid-soluble proteins from whole rabbit kidney

The electrophoresis was carried out as described in Fig. 1. The fractions stained with bromophenol blue in tannin reagent were eluted with 0.1 N-NaOH and protein determined by the tannin micromethod.

Expt. no.	Relative percentage				
	K_1	K_2	K_3	K_4	K_5
1	8.8	28.2	11.3	12.6	39.1
2	11.0	45.4	12.0	12.0	19.6
3	14.6	40.0	10.4	11.2	23.8
4	22.9	49.2	8.6	6.1	13.2
5	19.4	14.2	14.2	14.2	38.0
Mean	15.3	35.4	11.3	11.2	26.7
±S.D.	5.8	12.2	2.1	6.1	11.4

The electrophoretic resolution of SSA-soluble proteins of the subcellular fractions is presented in Fig. 1. The SSA-soluble proteins of nuclei (Fig. 1b) corresponded in mobility to fraction K_5, the proteins of mitochondria (Fig. 1c) to fractions K_4 and K_3, whereas those of the post-mitochondrial supernatant (Fig. 1d), to the slowest-moving fractions K_2 and K_1. Although the SSA-soluble proteins of nuclei

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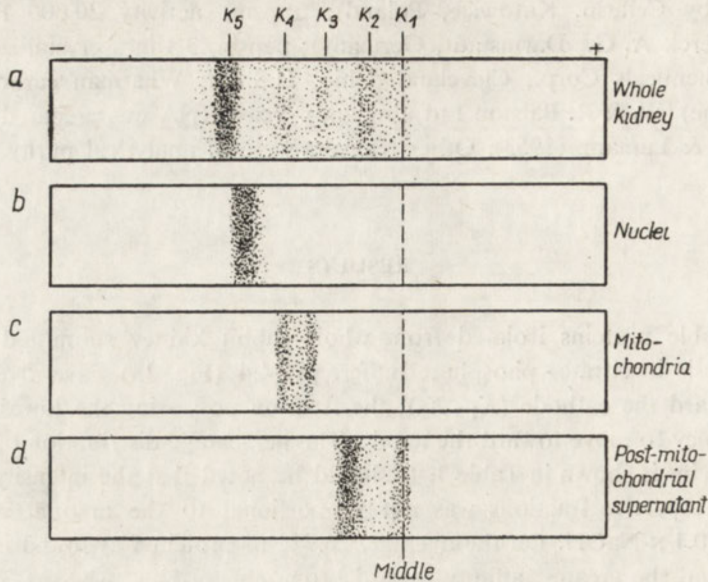

Fig. 1. Paper electrophoresis of sulphosalicylic acid-soluble proteins of whole rabbit kidney and subcellular fractions. Conditions of electrophoresis: 0.15 M-citrate - phosphate buffer, pH 4.4, time 6 hr., voltage 200 v. The protein (200 - 400 µg.) was applied at the middle of a strip of Schleicher-Schüll no. 2043a paper. The electrophoretograms were stained with bromophenol blue in tannin reagent.

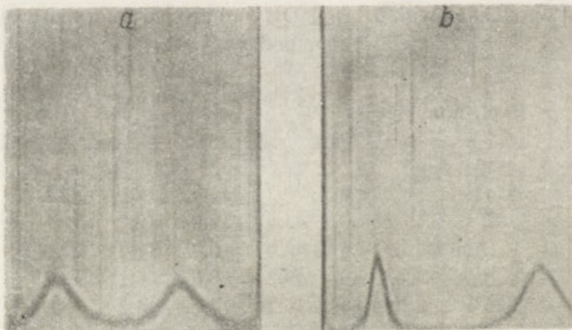

Fig. 2. Free-boundary electrophoresis of sulphosalicylic acid-soluble proteins of rabbit kidney nuclei. The electrophoresis was carried out in 0.15 M-citrate - phosphate buffer, pH 4.4, at 85 v, 3 mA, and 4°, for 2 hr.; (*a*), ascending part; (*b*), descending part.

migrated on paper electrophoresis as a single band, on free-boundary electrophoresis they appeared to be heterogeneous and separated into two symmetrical peaks moving toward the cathode (Fig. 2).

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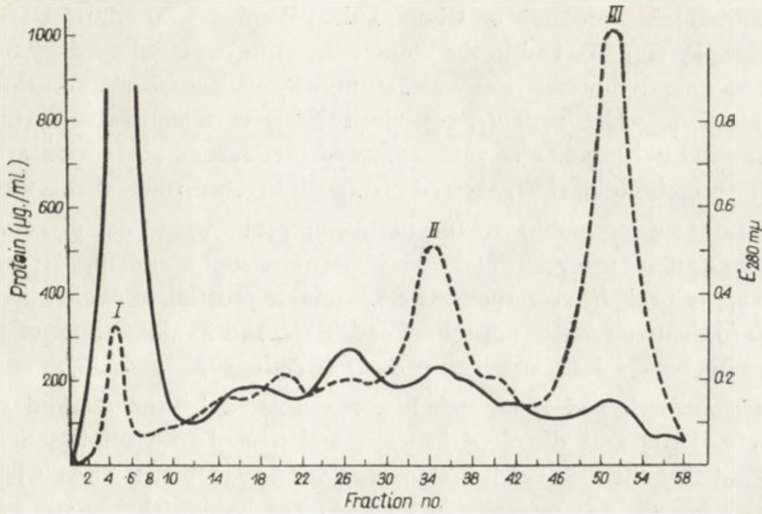

Fig. 3. Fractionation of sulphosalicylic acid-soluble proteins of whole rabbit kidney on CM-cellulose column (1 × 15 cm.). Fractions of 3 ml. were eluted with 0.05 M-acetate buffer, pH 4.2, containing from 0 to 0.6 M-KCl. (- - -), Protein assayed by the tannin method; (———), protein assayed spectrophotometrically.

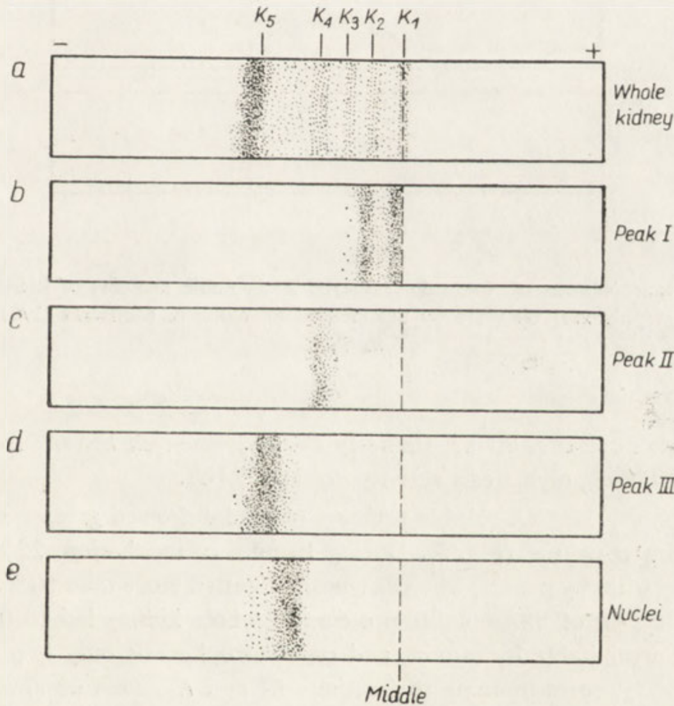

Fig. 4. Paper electrophoresis of whole kidney homogenate and the fractions obtained by CM-cellulose chromatography shown in Fig. 3. For comparison, sulphosalicylic acid-soluble protein of the nuclear fraction is also presented.

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The SSA-soluble proteins of whole kidney were submitted to CM-cellulose chromatography (Fig. 3), and in the eluates the protein was assayed by the tannin method and spectrophotometrically at 280 mμ. By the tannin method three peaks were obtained, of which peak *III* contained the greatest amount of protein, and peak *I* the smallest. Proteins of peak *I* showed pronounced absorption at 280 mμ, whereas the proteins of peak *III* showed practically no absorption at this wavelength.

The eluates corresponding to the particular peaks were pooled, concentrated by the tannin-caffeine procedure and submitted to electrophoresis (Fig. 4); it appeared that proteins of peak *III* correspond to SSA-soluble proteins of nuclei, the proteins of peak *II* to the two fractions of mitochondria (K_4 and K_3), and those of peak *I* to the post-mitochondrial supernatant protein (fractions K_2 and K_1).

The arginine-rich and lysine-rich histones isolated by the method of Wolfe & McIlwain (1961) were dissolved in water and treated with sulphosalicylic acid. It was found that the lysine-rich histone was soluble in 0.15 M-SSA, whereas the arginine-rich histone was precipitated by SSA. The lysine-rich histone, when submitted to paper electrophoresis at pH 4.4, exhibited the mobility of the SSA-soluble protein of nuclei (Fig. 5).

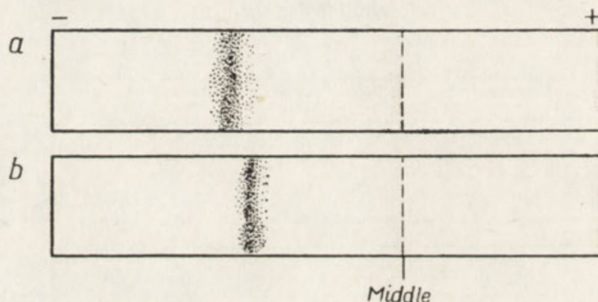

Fig. 5. Paper electrophoresis of: (*a*), sulphosalicylic acid-soluble proteins of kidney nuclei; (*b*), lysine-rich nuclear histone, obtained by the method of Wolfe & McIlwain (1961) and soluble in SSA.

SSA-soluble nuclear proteins, similarly as the lysine-rich histone fraction, were found to exhibit depolymerase activity toward RNA.

On proteolysis, the SSA-soluble proteins of nuclei showed greater susceptibility to trypsin than to pepsin (Fig. 6). During 10 min. of incubation, 25% of protein underwent digestion by pepsin, whereas trypsin digested more than 60%. Exhaustive digestion by trypsin of SSA-soluble proteins of whole kidney led to formation of products not precipitable by tannin, and on electrophoresis only two fractions of very low mobility, corresponding to fractions K_1 and K_2, were obtained (Fig. 7b). On the other hand, the exhaustive digestion by pepsin gave only a small decrease in the amount of peptide reacting with tannin, but the number of the electrophoretic fractions remained unaltered (Fig. 7c).

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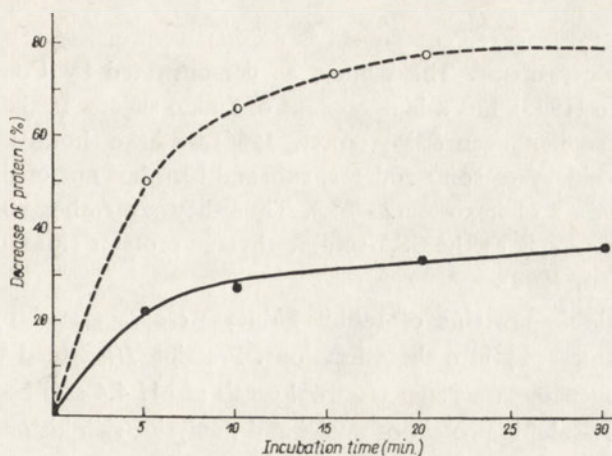

Fig. 6. Time-course of proteolysis of sulphosalicylic acid-soluble proteins of nuclei. The incubation mixtures contained in 1 ml. 100 µg. of protein and 1 µg. of (●), pepsin and (○), trypsin. The reaction was stopped by the addition of tannin reagent, and the undigested protein determined.

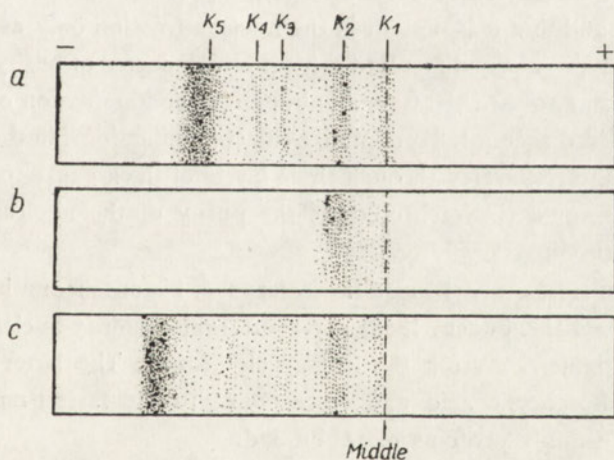

Fig. 7. Paper electrophoresis of polypeptides obtained by exhaustive digestion from sulphosalicylic-acid soluble proteins of whole rabbit kidney. (*a*), Prior to proteolysis; (*b*), after digestion by trypsin; (*c*), after digestion by pepsin. The incubation mixtures contained in 1 ml. 100 µg. of protein dissolved in 0.1 M-phosphate buffer, pH 7.6, and 5 µg. of trypsin; or 100 µg. of protein dissolved in 0.06 N-HCl and 5 µg. of pepsin. After 4 hr. at 30°, the mixtures were adjusted to pH 2 and the polypeptides were concentrated by the tannin-caffeine procedure and submitted to paper electrophoresis. Conditions of electrophoresis as in Fig. 1.

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DISCUSSION

The presence of a sugar component is a common property of most sulphosalicylic acid-soluble proteins. The kidney, as demonstrated by Comb & Roseman (1960) and Spiro (1965), has a high content of amino sugars. In the previous paper (Mejbaum-Katzenellenbogen & Wieczorek, 1966) we have shown that SSA-soluble proteins of the kidney of some rodents, calf and hen, but not of the frog, contain only small amounts of hexoses (2 - 7%). Thus they are rather polypeptides than glycopeptides, similarly as the SSA-soluble thymus proteins (Maskos & Mejbaum-Katzenellenbogen, 1968).

The SSA-soluble proteins of rabbit kidney were separated by CM-cellulose column chromatography into three fractions. Fraction *III*, eluted with 0.3 - 0.4 M-KCl, was homogeneous on paper electrophoresis at pH 4.4 and had the same mobility as the SSA-soluble protein of nuclei and the lysine-rich histone isolated from nuclei. A similar resolution on CM-cellulose column of the SSA-soluble protein of hog kidney was reported by Rzeczycki *et al.* (1962, 1963); however, in their experiments the protein of fraction *III* on paper electrophoresis at pH 8.6 had the mobility of the SSA-soluble mitochondrial protein. This fraction, called by the authors fraction A, was lysine-rich as demonstrated by amino acid analysis. Some protein corresponding to fraction A could be extracted with SSA from the nuclear fraction, but its amount was small and greatly diminished after exhaustive washing of the nuclei. Therefore Rzeczycki concluded that the lysine-rich fraction A is of mitochondrial origin and that it is present in the nuclear fraction only as a contamination. Undoubtedly, it is very difficult to achieve a high degree of purity of subcellular fractions. According to the literature, an about 10% contamination of each fraction should be taken into account. To minimize the contaminations, in the present work the homogenates were filtrated through three layers of thick gauze to separate them from non-disintegrated tissue. Moreover, the purity of the nuclear fraction was checked by microscopy.

Application of sulphosalicylic acid for isolation of histones from thymus (Maskos & Mejbaum-Katzenellenbogen, 1968) and from rabbit kidney nuclei, permitted to separate the arginine-rich from the lysine-rich histone. The latter is extractable with 0.15 M-sulphosalicylic acid and corresponds to the fastest-moving cathodic fraction of SSA-soluble proteins of rabbit kidney.

This work was supported in part by a grant from the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences.

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BIAŁKA ROZPUSZCZALNE W KWASIE SULFOSALICYLOWYM ORAZ ICH LOKALIZACJA WE FRAKCJACH PODKOMÓRKOWYCH NERKI KRÓLIKA

Streszczenie

1. Z jąder komórkowych nerki królika wyizolowano histony bogate w lizynę, które ekstrahowały się do 0,15 M-kwasu sulfosalicylowego i odpowiadały w elektroforezie bibułowej frakcji najbardziej katodowej białek rozpuszczalnych w kwasie sulfosalicylowym nerki królika oraz białkom szczytu III-go w chromatografii na karboksymetylocelulozie. Frakcja ta wykazuje aktywność depolimerazy RNA oraz trawi się trypsyną.

Received 14 June, 1968.

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RECENZJE KSIĄŻEK

REGULATION OF METABOLIC PROCESSES IN MITOCHONDRIA (J. M. Tager, S. Papa, E. Quagliarello & E. S. Slater, eds.). Elsevier Publ. Comp., Amsterdam, New York, London 1966; str. 586. cena 75 fl.

Omawiana książka, stanowiąca VII tom biblioteki B.B.A., zawiera wykłady i dyskusje przeprowadzone na Sympozjum pod wyżej wymienionym tytułem. Sympozjum to odbyło się w Bari (Italia) jako wspólna impreza Zakładu Biochemii tamtejszego uniwersytetu oraz Laboratorium Biochemii uniwersytetu w Amsterdamie. Wygłoszono 34 referaty, których teksty, wraz z całą dokumentacją oraz dyskusją, stanowią treść książki. Nie sposób w krótkiej recenzji omówić szczegółowo lub choćby wymienić tylko tytuły referatów, podam więc tylko w zarysie najważniejsze omawiane tematy. Omówiono więc ultrastrukturę mitochondriów, enzymy i substraty najważniejszych procesów metabolicznych, zagadnienie kompartmentacji, pęcznienia i kontrakcji mitochondriów, zagadnienie syntezy białka w mitochondriach, wpływ hormonu tarczycy na metabolizm mitochondriów i in. Wiele miejsca, zwłaszcza w dyskusji, zajęła sprawa magazynowania i przenoszenia energii, przy czym dyskutowano obszernie chemiosmotyczną teorię Mitchella, konfrontując ją przy różnych okazjach z ogólnie przyjętą teorią przenośników chemicznych. Obszernie omawiano też mechanizm działania atraktylozydu, który hamuje fosforylację oksydatywną w ostatnim jej etapie, przy powstaniu ATP z ADP i P_i.

Referaty, a zwłaszcza żywa dyskusja, dały jasny obraz współczesnej wiedzy o mitochondriach, uwypuklając różnice w interpretacji obserwowanych faktów przez różnych badaczy. Podkreślono niebezpieczeństwa wynikające z drobnych odchyleń w warunkach doświadczenia.

Ostatni dzień Sympozjum poświęcono dyskusji nad wybranymi zagadnieniami, które wyłoniły się w trakcie Sympozjum.

Dla czytelnika polskiego książka jest tym ciekawsza, że dalszy ciąg dyskusji, w nieco szerszym gronie, odbył się w rok później w Warszawie w ramach zebrania FEBS'u.

Józef Heller

MAGNETIC RESONANCE IN BIOLOGICAL SYSTEMS (A. Ehrenberg, B. G. Malmström and T. Vänngård, eds.). Symposium Publications Division, Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig 1967; str. 431, cena 7 *Ł*.

Jakkolwiek już Faraday w roku 1845 robił doświadczenia nad magnetycznymi własnościami suszonej krwi, to jednak zjawiska rezonansu magnetycznego zaobserwowano dopiero w sto lat później i wkrótce zastosowano je do badania własności jąder, atomów i cząsteczek. Wyrazem znacznych osiągnięć, jakie udało się uzyskać w badaniu układów biologicznych przez zastosowanie spektroskopii magnetycznego rezonansu jądrowego (NMR) i elektronowego rezonansu paramagnetycznego (EPR), jest omawiana książka.

Jest to zbiór referatów wygłoszonych w czasie sympozjum, jakie odbyło się w czerwcu 1966 r. w Centrum Wenner-Grena w Sztokholmie. Książka zawiera 50 referatów dotyczących oryginalnych badań przy użyciu powszechnie stosowanej aparatury do spektroskopii NMR i EPR; pewna liczba prac dotyczy technicznych modyfikacji i ulepszeń stosowanej techniki, a w końcowych pięciu re-

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feratach opisano doświadczenia, w których wykorzystano do badania układów biologicznych efekt Mössbauera. Czytelnik dowiaduje się z omawianej książki, że spektroskopię rezonansu magnetycznego wykorzystano nie tylko do badania centrów aktywnych enzymów, mechanizmów niektórych reakcji i konformacji cząsteczek, ale także do badania prędkości bardzo szybkich reakcji, do ustalania sekwencji w peptydach i in. Niewątpliwie słuszna jest uwaga Hugo Theorella, wypowiedziana w jego wstępnym przemówieniu w czasie trwania sympozjum, że badanie magnetycznych własności cząsteczek stworzyło nową gałąź wiedzy, którą nazwać by można „biologią submolekularną".

Jedynie przy niewielu referatach zamieszczono krótkie komentarze wygłoszone w dyskusji, ale ze zdjęcia zamieszczonego na końcu książki, a przedstawiającego dyskutantów na tle pięknej rzeźby Millesa, można wnosić, że dyskusje na sympozjum były ożywione.

Mariusz Żydowo

ADVANCES IN ENZYME REGULATION (G. Weber, ed.) vol. 5. Pergamon Press, Oxford 1967; str. 469, cena $ 20.

Omawiany tom przynosi materiały kolejnego Sympozjum na temat regulacji enzymatycznej, które odbyło się w Indiana-University. Na Sympozjum złożyło się 11 posiedzeń, z jednym do trzech referatów na każdym i z dyskusją. Rozpoczął B.Chance przedstawiając wypracowaną przez swoich współpracowników metodę mikrofluorymetrii różnicowej. O. Haiaishi mówił o biosyntezie NAD *in vivo*, po wprowadzeniu do wątroby znakowanych prekursorów w rytmicznym dawkowaniu.

W następnym posiedzeniu, Wiliams-Ashman przedstawił pracę zbiorową nad wpływem testosteronów na aktywność DNA-polimerazy w prostacie, a J. M. Fain mówił o roli syntezy białka w działaniu lipolitycznym hormonu wzrostowego.

Na posiedzenie trzecie złożyły się referaty N. Katunuma o regulacji metabolizmu glutaminowego w nerce i L. Goldsteina o wspólnych z J. N. Schoolerem badaniach nad regulacją powstawania amoniaku w nerce szczura.

Posiedzenie czwarte, poświęcone regulacji metabolicznej drogą biosyntezy enzymów, obejmowało referaty: D. Connor-Johnsona i H. S. Sassoona o indukcji wątrobowej dehydrogenazy glukozy 6P, H. J. Fallona o regulacji przemiany seryny u ssaków, oraz C. G. Smitha i współpracowników o biochemicznym i biologicznym działaniu tubercydyny (7-Deaza-adenozyny) i pokrewnych związków.

Posiedzenie piąte wypełniły referaty F. Chapeville i P. Fromageot o „śladowych" enzymach w embriogenezie, oraz Sereni'ego o polimerazie RNA w embriogenezie szczura. „Śladowe" enzymy, to enzymy metabolizmu siarki i aminokwasów siarkowych, które u ssaków dorosłych w znacznym stopniu zanikły.

Na posiedzeniu szóstym M. Suda przeprowadził porównanie regulacji enzymatycznej u ssaków i drobnoustrojów, a H. Holzer przedstawił badania swojej grupy nad hamowaniem syntetazy glutaminowej w *E. coli* przez metabolity.

Glukoneogenezie poświęcono posiedzenie siódme, z referatami J. K. Williamsona o wpływie kwasów tłuszczowych, glukagonu i surowicy anty-insulinowej, oraz Webera i współpracowników (gospodarze Sympozjum) o mechanizmach kontrolujących aktywność enzymów.

Ósme posiedzenie poświęcono działalności hormonów *in vitro*. V. R. Potter omawiał badania swojej grupy nad wpływem hormonów na aktywność enzymatyczną w hodowli tkankowej *in vivo*, zaś A. White przedstawił badania nad wpływem glukokortykoidów na tymocyty *in vitro*.

Na posiedzeniu dziewiątym R. E. Olson omawiał regulacyjną funkcję witaminy E, zaś H. M. Katzen mówił o różnorodności heksokinazy ssaków. Ciekawe rozważania teoretyczne nad pojęciem „Feedback" zawierał referat D. S. Rigsa.

Posiedzenie dziesiąte z referatami Gelboina i współpracowników o wpływie biostatyków na RNA-polimerazę i Greengarda nad zachowaniem się enzymów u szczurów głodzonych, naświetlanych i traktowanych glukagonem, zamykało „normalny" program Sympozjum.

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Osobną specjalną sesję stanowił referat H. A. Krebsa o stanie redoxowym NAD w cytoplazmie i mitochondriach wątroby szczura.

Charakter omawianego tomu nie odbiega od poprzednich tej serii, zdradzając indywidualny wpływ organizatora sympozjów i wydawcy *Advances*, G. Webera. Ostatnie Sympozjum programowo zawężono do badań nad ssakami. Tom zdobi fotografia uczestników Sympozjum.

Józef Heller

UREA AS A PROTEIN SUPPLEMENT (Michael H. Briggs, ed.).Pergamon Press, Oxford, 1967; str. 466, cena 50 s, $ 8.00.

Szybki przyrost ludności oraz towarzyszący mu problem zaopatrzenia w białko jest jednym z ważniejszych zagadnień współczesnej nauki. Omawiana książka jest zbiorem referatów poświęconych wykorzystaniu przez zwierzęta syntetycznego mocznika jako źródła azotu białkowego.

Książka składa się z czterech części, w których omówiono kolejno metody syntezy mocznika, jego wpływ na procesy fizjologiczne zwierząt, oraz możliwości przyswajania go przez zwierzęta przeżuwające i nieprzeżuwające.

W części pierwszej przedstawiono historię badań nad syntezą mocznika i omówiono obszernie stosowane na świecie metody jego produkcji.

Metabolizm związków azotowych niebiałkowych w przewodzie pokarmowym zwierząt przeżuwających oraz wpływ różnych czynników na te procesy jest tematem drugiej części książki. Przedstawiono tu szczegółowo wyniki badań nad metodami podawania mocznika; omówiono jego wpływ na metabolizm bakterii przewodu pokarmowego; zreferowano obszernie udział związków azotowych w syntezie białek mleka i wykazano, że mają one taki sam skład jak białka mleka zwierząt przy normalnej diecie.

Dokładna analiza warunków podawania mocznika zwierzętom przeżuwającym, a w szczególności krowom, wpływ diety na jego przyswajanie, toksyczność oraz metabolizm są tematem rozważań trzeciej części książki.

Czwarty i ostatni rozdział poświęcony jest badaniom nad przyswajaniem mocznika przez zwierzęta z grupy nieprzeżuwających. Na podstawie fragmentarycznych danych i badań bardzo jeszcze wstępnych wydaje się, że świnia i kurczęta nie wykorzystują azotu mocznika do syntezy własnych białek.

Książka *Urea as a Protein Supplement* zawiera wiele ciekawych danych odnośnie przyswajania azotu niebiałkowego przez zwierzęta, zawiera też bogaty zbiór piśmiennictwa. Książka stanowić może interesującą i pomocną lekturę zarówno dla teoretyków, jak i praktyków zajmujących się doświadczalną hodowlą bydła.

Zofia Porembska

TESTOSTERONE. Proceedings of the Workshop Conference held from April 20th to 22nd, 1967, at Tremsbuttel (J. Tamm, ed.). G. Thieme Verlag, Stuttgart 1968; str. 246, cena 48 DM.

Omawiana książka stanowi dokładne sprawozdanie z konferencji poświęconej testosteronowi. Konferencja zgromadziła doświadczonych badaczy z zakresu endokrynologii i biochemii sterydów, wśród których widnieją tak świetne nazwiska jak Baulieu, Bush, Dorfman, Eik-Nes, Lipsett, Neher, Peterson, Samuels, Vermeulen i Wettstein.

Tematycznie konferencja została podzielona na siedem Sesji: metodologia, biochemia, fizjologia, antyandrogeny, aspekty kliniczne u mężczyzn, aspekty kliniczne u kobiet i dyskusja ogólna (podsumowująca). Dwie pierwsze sesje były najbogatsze, a sprawozdania zajmują połowę objętości książki. Materiał ten przedstawia rzeczywisty, ogromny postęp metodyczny, łącznie z badaniami na związkach znakowanych, rozdzielanych we wszystkich technikach chromatograficznych, wraz z gazową, jak również postęp w biochemii sterydów wynikający z rozwoju techniki.

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W sprawozdaniu z każdej sesji (oprócz ostatniej) są przedstawione referaty w przyjętej formie wyczerpujących publikacji, łącznie z aktualnym piśmiennictwem oraz obszerną, bardzo ciekawą, przytoczoną dosłownie dyskusją. Dyskusja ta, obrazująca ustosunkowanie się do referowanego zagadnienia najwybitniejszych znawców, stanowi najcenniejszą część sprawozdania.

Merytorycznie książka gromadzi wyniki najbardziej nowoczesnych doświadczeń i przedstawia współczesny dorobek ostatnich 4 - 5 lat badań nad testosteronem. Jest ona bardzo dobrym przeglądem wiadomości z zakresu teoretycznej i klinicznej endokrynologii, których często nie ma jeszcze w fachowej literaturze.

Według opinii recenzenta daje się zaobserwować duża dysproporcja pomiędzy postępem technicznym, dzięki któremu można precyzyjnie oznaczać testosteron lub jego pochodne w ilościach rzędu milimikrogramów (nanogramów), a przydatnością diagnostyczną tych oznaczeń; np. nadal nie znamy przyczyny wielu postaci nadmiernego owłosienia u kobiet.

Barbara Migdalska

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